Spatial and temporal distribution of growth factors and their receptors in diabetic retinopathy

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ABSTRACT

PURPOSE

To determine the distribution of vascular endothelial growth factor (VEGF) isoforms, angiopoietins (Ang-1 and -2) and their receptors, tumour necrosis factor alpha (TNF-α), pigment epithelium-derived factor (PEDF), and caveolin family members (Cav-1, -2, and -3) in non-diabetic retinas and diabetic retinas at different stages of diabetic retinopathy.

METHODS

Human eyes, obtained at post-mortem, were divided into those without diabetes and those with diabetes. Diabetic retinas were examined by microscopy and categorised as either non-lasered with no obvious features of retinopathy, non-lasered with intraretinal changes (microaneurysms, exudates etc.) but no evidence of proliferative diabetic retinopathy (PDR), diabetic with proliferative retinopathy, and those which had received scatter laser photocoagulation therapy and who no longer had evidence of PDR. Immunohistochemistry was used to determine the localisation of growth factors and caveolins in diabetic and control retinas, as well as in excised PDR membranes.

RESULTS

There appeared to be both temporal and spatial changes in the staining pattern for each growth factor in diabetic retina which correlated with the stage of disease progression. Apart from Ang-2 and PEDF, immunostaining was raised in diabetic retina as compared to non-diabetic retina. Immunostaining was apparent in endothelial cells and the perivascular cells of the vessels. Immunostaining was also apparent within specific retinal layers for VEGF-A, VEGF-C, VEGFR's, Ang-2, Tie-2, TNF-α, PEDF, and caveolin 1, -2, and -3.

CONCLUSION

These data suggest a role for both angiogenic factors and anti-angiogenic factors and the caveolins in the pathogenesis of diabetic retinopathy, possibly by acting synergistically to mediate a wide range of cellular responses culminating in the formation of a fibrovascular membrane. Therapeutic intervention to the VEGF and Tie-2 receptor, and possibly stimulation of the PEDF signalling, pathways may prove useful for the treatment of PDR.
CHAPTER 1. INTRODUCTION

1.1 GENERAL INTRODUCTION

Diabetes mellitus is a metabolic disorder characterised by hyperglycaemia and alterations in fat and protein metabolism and is associated with a specific set of long term microvascular and neurological complications (Nathan, 1996). World-wide it is estimated that over 2.5 million people in the 25-65 year age group are blind due to diabetes, which makes it the fourth leading cause of blindness and an increasing problem in developing nations (Foster, 1988). Typical ocular complications of diabetes include diabetic retinopathy, iris neovascularization, glaucoma, cataract and microvascular abnormalities of the optic nerve (Infeld et al., 1998). The most frequent complication is diabetic retinopathy in which retinal ischaemia, occurring as a consequence of widespread capillary non-perfusion, results in the production of vasoproliferative substances and the development of neovascularization (Infeld et al., 1998). Signs of retinopathy are not usually manifest before duration of 15 to 20 years, at which time the prevalence can approach 80% to 100% in insulin-dependent diabetics (Frank, 1986).

1.2 DIABETES MELLITUS AND ITS COMPLICATIONS

Diabetes mellitus is associated with typical patterns of long term vascular complications which vary with the organ involved. The microvascular kidney disease (Olgemoller et al., 1993) is characterised by thickening of the capillary basement membranes and increased deposition of extracellular matrix components, while loss of microvessels with subsequent neovascularization is predominant in the eye and peripheral nerves (Pfeiffer et al., 1995). Macrovascular disease is characterised by accelerated atherosclerosis. These complications are dependent on long term hyperglycaemia. Specific biochemical pathways linking hyperglycaemia to microvascular changes have been proposed: the polyol pathway (Greene et al., 1987), non-enzymatic glycation of proteins (Brownlee et al., 1988), glucose autooxidation and oxidative stress (Hunt et al., 1990), hyperglycaemic pseudohypoxia (Williamson et al., 1993), and enhanced activation of protein kinase C by synthesis of diacetyl glycerol (Lee et al., 1989; DeRubertis and Craven, 1994). These pathways are not mutually exclusive (Larkins and Dunlop, 1992; Pfeiffer and Schatz, 1992). They may be linked to alterations in the synthesis of growth factors particularly since atherosclerosis and angiogenesis are associated with increased proliferation of endothelial cells.

Diabetic retinopathy may be classified as nonproliferative (NPDR) or proliferative (PDR) [Neely et al., 1998]. NPDR may be graded as mild, moderate, or severe. The level of
severity correlates with the probability of progression to PDR (Neely et al., 1998). NPDR is characterised by structural abnormalities of the retinal vessels (primarily capillaries but also venules and arterioles), varying degrees of retinal nonperfusion, retinal oedema, lipid exudates, and intraretinal haemorrhages. An important pathological event is the loss of capillary pericytes which are modified smooth muscle cells, which support the vascular endothelium of the retina (Kohner, 1993). This appears to be the initial event in NPDR. PDR may include any of the changes present in nonproliferative disease with additional findings of optic disc, retinal or iris neovascularization (Neely et al., 1998).

Both NPDR and PDR may cause visual loss with the major vision-threatening complications being macula oedema, macula ischaemia, neovascularization with preretal or vitreous haemorrhage, traction retinal detachment, and neovascular glaucoma (Neely et al., 1998). Before considering the pathogenesis of diabetic retinopathy in detail it is important to have a basic knowledge of retinal anatomy and physiology.

1.3 STRUCTURE AND FUNCTION OF THE NON-PATHOLOGICAL RETINA

The retina lines the posterior two-thirds of the eyeball (figure 1.1) and is separated from the sclera by the retinal pigment epithelium and the choroid (Naumann et al., 1986). The retina is firmly attached to the underlying pigment epithelium at the optic nerve head and the ora serrata.

Light rays, scattered from objects in the outside world, enter the eye and hit the retina. Some processing of images then occurs in the retina, from which electrical impulses are then transmitted through the optic nerve to the primary and secondary visual centres of the brain.

The eye is normally directed so that the image of an object falls upon the fovea centralis the central portion of the macula, a depression in the retina situated about a millimetre or so to the lateral side of the posterior pole of the eye formed by lateral displacement of the cells of the inner retinal layers (Emslie-Smith et al., 1988). It serves the function of central and colour vision and is therefore susceptible to many diseases of the retina such as diabetic retinopathy.
At the microscopic level, the retina is a highly organised structure, consisting of alternate layers of cell bodies and synaptic processes. A single common pathway, mediated by the retinal ganglion cells, carries information from the retina to the brain (Miller, 1994). The retina can be classified into three systems, the neuronal system, the glial system and the vascular system (Blanks, 1994). These three systems are located within the 9 layers of the retina (figure 1.2) and are described below.

1.3.1 The Neuronal System of the Retina

The neuronal system traverses the entire thickness of the retina and consists of photoreceptor cells, intermediate neurones and ganglion cells (Naumann et al., 1986).

The photosensitive cells, the rods and cones, lie in the outermost layer of the retina next to the pigment epithelium and serve the function of light perception (Emslie-Smith et al., 1988). Light rays pass through the ganglion cells and inner retinal layers to reach the photoreceptor cells, where light is transformed into an electrochemical event (Blanks, 1994).

Each photoreceptor consists of an outer segment and an inner segment, both of which lie external to the external limiting membrane (ELM). Each photoreceptor also
contains a cell body, together which form the outer nuclear layer (ONL). The processes of both rods and cones interact with the axons of the bipolar cells and horizontal cells in the outer plexiform layer (Naumann et al., 1986). The rods are thin, cylindrical structures which are primarily responsible for peripheral vision and vision in low illumination (scotopic vision). The cones, which in histological sections are distinguished by their broader flask-shaped profiles, are primarily responsible for phototopic vision and for highly discriminatory central and colour vision.

The cell bodies of four cells can be distinguished in the inner nuclear layer (INL): the most numerous of these are the bipolar cells. Their processes synapse outwardly with the photoreceptors, forming the middle limiting "membrane," and inwardly with the dendrites of the ganglion cells (Naumann et al., 1986).

Three types of bipolar cells have been distinguished morphologically. One morphologic type exclusively relates to rods, and two types relate exclusively to cones. The two cone-related types of bipolar cells make different kinds of synaptic connections with the cones, have axonal terminals that end in different parts of the inner plexiform layer, and appear to be involved in the generation of either ON or OFF responses to light in the retina. Rod-related bipolar cells extend their dendrites into invaginations of the rod terminals. The dendritic ends of the rod bipolar cells are considerably larger than those of the cone bipolar cells and allow a single rod bipolar cell to contact as many as 45 rod terminals.

The horizontal cells are located at the outer region of the INL. The horizontal cells (and amacrine cells which are discussed below) provide numerous "horizontal" neural
interconnections between groups or fields of retinal sensory neurones. In the retina, the processes of horizontal cells (and amacrine cells) modulate and transform visual information that is conveyed to the brain.

In the primate retina there are two morphologically distinct types of horizontal cells (Blanks, 1994). The type I horizontal cell is characterised by stout dendrites that contact only cones, and a single, long axon ending with a terminus that contacts only rods. These dendritic terminals form the lateral elements of the rod synapse. The type II horizontal cell contacts only cones with its slim dendritic branches and short axon.

Amacrine cells are located primarily in the inner portion of the inner nuclear layer. They extend processes to adjacent amacrine or bipolar cells, and their axons synapse with ganglion cells within the inner plexiform layer. Displaced amacrine cells are also present within the ganglion cell layer (GCL). Amacrine cells are more diversified and numerous than horizontal cells.

Until recently, amacrine cells have been classified according to the degree of stratification of their processes, e.g., as unstratified, bistratified, or multistratified if many branches of the main processes ramify in one, two, or more levels, respectively, or as diffuse if the many branches ramify without stratification. In the last several years, classification of amacrine cells, based on their neurotransmitters, has changed dramatically because of the finding that there may be as many as 30 different types. It has been shown that amacrine cells with differently shaped dendritic trees can be matched with particular neurotransmitters.

Interplexiform cells were recognised only 15 years ago as a distinct class of neurones in the vertebrate retina. This interneurone has a cell body located at the innermost border of the INL, but its processes extend into both the inner and outer plexiform layers.

Nasally, the GCL consists of a single row of cells, which are separated by the processes of the Müller fibres (Naumann et al., 1986). They are closely grouped near the optic nerve but are widely separated in the periphery: they are only occasionally seen at the region of the ora serrata. Temporally from the nerve, the ganglion cells become multilayered, increasing to six to eight layers of cells in the macula region.

The axons of ganglion cells converge to form the nerve fibre layer and exit the eye as the optic nerve. Ganglion cells and to a lesser extent bipolar cells, can have quite extensively spreading dendrites so that each ganglion cell may be influenced by the activity of a large number of rods and cones. Further lateral interactions are mediated by the horizontal and amacrine cells. In all regions of the retina, except the central fovea, the ganglion cells can be seen to be connected, through intermediate bipolar cells, to rods as well as cones.
1.3.2 The Glial System of the Retina

The glial system of the retina consists of Müller Cells, astrocytes and microglia. Müller cells are the most prominent of all retinal cells and they stretch from the internal limiting membrane (ILM) to beyond the ELM (Naumann et al., 1986). The Müller cell bodies lie in the inner two thirds of the INL, among the cell bodies of the amacrine and bipolar cells. Müller cell radial fibres are prominent in the inner retina, where the main ascending processes are thick and relatively straight. They pass directly through the inner plexiform layer, among the ganglion cells, and between the nerve fibre bundles where they terminate internally on the outer surface of the ILM by formation of basal foot processes. Many delicate horizontal processes extend laterally from these radial fibres, which are particularly prominent as the horizontal fibres of the nerve fibre, inner and outer plexiform layers. In the nuclear layers, these lateral processes form a honeycomb around the various cell bodies. Müller cell processes intervene between most smaller vascular elements and neuronal processes. In the outer retina the ELM is formed by a network of cell junctions between Müller cell processes and photoreceptors.

In contrast to most other elements of the retina, which possess either a photoreceptive or neurotransmission function, the Müller cell provides structural support and contributes to the metabolism of the sensory retina. The cytoplasm of Müller cells has a high content of glial fibrils, glycogen granules, and abundant smooth endoplasmic reticulum. It also contains abundant lactic acid dehydrogenase suggesting they have an important role in carbohydrate metabolism in the retina. Müller cells also may be active in the degradation of synaptic transmitters such as Gamma amino butyric acid (GABA) and in regulating extracellular levels of glutamine released during neuronal activity. Another important function of Müller cells concerns the maintenance of potassium homeostasis in the retina. Neuronal activity is associated with the release of potassium into the extracellular space. Potassium is removed from the outer retina, probably by an active pump located in the Müller cell villi, and leaves these cells most likely with the aid of an active pump located in the endfoot membrane.

Fibrous astrocytes, typical of those found elsewhere in the central nervous system (CNS), are common in the nerve fibre layer of the retina where their number is proportional to the thickness of the layer. There are 2 types of astrocytes present in the retina, elongate astrocytes and stellate astrocytes. Elongate astrocytes have multiple long slim processes that traverse the retina within the nerve fibre bundles. They have not been observed to contact blood vessels. Stellate astrocytes have many slender processes that cross the nerve fibres; some of these processes contact nearby blood vessels with a bulbous endfoot. Astrocytes are
found occasionally at other locations in the inner half of the retina, but with much less frequency than the nerve fibre layer. Blood vessels never directly contact neuronal processes; intervening processes may be astrocytic but are most commonly derived from Müller cells.

Microglia contain an elongate nucleus and a slender cell body with two or more thick basal processes from which multiple coarse, short secondary processes may branch. The entire cell is covered with blunt spines from which slender hair-like extensions may continue 10-25 microns from the cell. The cells tend to be flat and are most common in the inner plexiform layer but they also occur in the external plexiform layer. Microglia are mesodermal in origin and are thought to arise from pericytes of blood vessels. As in the brain, they are amoeboid, migrate freely, are phagocytic, and become activated under pathological conditions of the retina.

The retinal pigment epithelium (RPE) is composed of a single-layered cuboidal or cylindrical epithelium containing pigment granules, a nucleus, mitochondria, and other cell organelles that mediate the active metabolic, fluid exchange, and phagocytic functions of the cells. (Naumann et al., 1986). Because the sensory-pigment epithelium attachment is firm only at the ora serrata and optic nerve, the retina is susceptible to pathological separation from the underlying pigment epithelium.

1.4 VASCULAR SUPPLY TO THE RETINA

The primary arterial supply to the eye is the ophthalmic artery, which is the first branch of the internal carotid artery. The ophthalmic artery enters the orbit with the optic nerve through the optic canal and then divides into two major subdivisions: (1) the retinal system, in particular the central retinal artery, which supplies the inner one-half of the retina; and (2) the ciliary system, which supplies the uvea as well as the outer half of the sensory retina and optic nerve (Naumann et al., 1986).

The secondary and tertiary branches of the central retinal artery occurring on the optic nerve head, as well as more peripherally in the fundus, form the arterioles and the primary blood supply to the four quadrants of the fundus. In a small percentage of cases a cilioretinal artery is present. This vessel originates from the short posterior ciliary vessels (the circle of Zinn) and enters the superficial aspect of the retina at the margin of the optic nerve. The four primary fundus arterioles lie superficially in the nerve fibre layer. The arterioles can easily be distinguished from the venules- not only clinically by their colour differences and smaller diameter but also histologically by evaluation of the vessel wall thickness. The terminal
fundus arterioles bend sharply and dip almost vertically into the retina, forming a rich capillary network. The capillaries extend as deeply as the outer aspect of the INL, sometimes extending just into the outer plexiform layer to the dividing line formed by the middle limiting "membrane".

The central fovea is almost totally devoid of blood vessels; however, the parafoveal and perifoveal zones or the more peripheral macula zones are richly vascularized by three arcades of capillaries within the inner half of the retina. Most of the extramacula and extrapapillary fundus is supported by two layers of capillaries; peripherally, this is reduced to a scanty single layer as the ora serrata is approached.

The branches of the retinal vein run alongside the arteries, the retinal vein passing out of the eye in the optic nerve (Emslie-Smith et al., 1988). A separate choroidal system of vessels lies between the pigment epithelium and the sclera and supplies the outer layers of the retina.

The retinal capillaries consist of two distinct cell types: the endothelial cell (EC) and the pericyte (PC) (Naumann et al., 1986). ECs line the lumen of the capillary and in turn are encircled by their basement membranes (BM) [figure 1.3].

![Figure 1.3 Normal Retinal Capillaries. a. Flat Preparation. b. Cross Section Showing The Nonfenestrated Endothelial Lining. (Copied from Naumann et al., 1986)](image)

The ECs of a normal retinal capillary are closely bound together about the lumen by intercellular junctions of the zonula occludens type (tight junctions). These inter-cellular bridges render the vascular channels nonfenestrated and thus prohibit a free flow of fluids from the vascular lumen into the retinal interstitium outside the capillary. These junctions, in
association with the astrocyte and Müller cell foot processes that encircle the retinal capillaries, probably contribute to the blood-retina barrier. Newly formed vessels (e.g. neovascularization in diabetic retinopathy) characteristically leak dye after fluorescein angiography, perhaps because of qualitatively and/or quantitatively incomplete intercellular tight junctions.

In addition to regulating vascular permeability ECs have a number of other functions. They are responsible for preserving vascular integrity with respect to maintaining uninterrupted blood flow by inducing platelet aggregation to plug injured sites and by triggering the extrinsic pathway of clotting. They are involved in the inhibition of intravascular thrombosis and lysis of established clots. They participate in the control of vascular tone, both vasodilator and vasoconstrictor substances are produced within the endothelium, e.g. nitric oxide (NO) [Garner, 1994] being a potent mediator of the first of these functions and endothelin-1 an equally effective mediator of the second.

ECs also have an inflammatory role in terms of leukocyte binding through the expression of specific adhesion molecules. The endothelium also can express class II major histocompatibility complex antigens and function as an antigen-presenting tissue in immunological reactions. It is a source of BM constituents and other extracellular proteins, such as fibronectin and a source of growth factors involved in angiogenesis and wound repair.

The PCs form a "cap" around the outer aspects of the EC membrane. Each cell extends processes that envelop the capillary and are sandwiched between layers of BM; in the retina about 85% of the lining endothelium is covered in this way. Various diseases cause a relative decrease in the number of pericytes. The most important of these is diabetic retinopathy.

The basic component of vascular BM is type IV collagen, which acts as a structural backbone and binds to other membrane components; it also has an inhibitory effect on EC proliferation, such that BM dissolution is obligatory before new vessel formation can take place. Laminin is another major constituent relating primarily to cell attachment, whereas fibronectin has a wider range of binding capacity. Proteoglycans, with heparin sulphate proteoglycan being the major one present and, due to the presence of their anionic charges, are crucial to the selective barrier function of the membranes.

The retinal vessels are also surrounded by perivascular glial elements, consisting of astrocytic foot processes and Müller cell processes (Naumann et al., 1986).
1.5 DIABETIC RETINOPATHY

Angiogenesis, the formation of new capillary blood vessels by sprouting from existing microvessels to promote neovascularization, plays a major role in the evolvement of a vascular supply in the normal processes of ovulation, placental development, and wound healing, as well as variously clinically significant pathological processes such as tumour growth and diabetic retinopathy (Ferrara, 1995a; Ferrara et al., 1995b). In contrast, the development of new vessels from blood islands, i.e., committed stem cells, in early embryogenesis is termed vasculogenesis. Although different in many respects, vasculogenesis shows some similarities to angiogenesis. However, vasculogenesis does not seem to contribute to repair and disease in postembryonic life (Battegay, 1995). In some of these processes the formation of new vessels contributes to necessary reparative processes involving tissue remodelling or preservation of vitally important ischaemic tissue. In other situations, such as in diabetic retinopathy, angiogenesis can significantly add to tissue destruction and promote disease (Battegay, 1995).

Many of the most sight-threatening ophthalmic disorders affecting patients throughout the world involve intraocular neovascularization as their major destructive component (Klein and Moorehead, 1970). Such disorders include not only the leading cause of blindness among infants (retinopathy of prematurity) [ROP], the leading cause of blindness among working age individuals (diabetic retinopathy), and the leading cause of blindness among the elderly (age-related macula degeneration), but other conditions such as central retinal vein occlusion, rubeosis iridis, sickle cell retinopathy, radiation retinopathy etc. (Klein and Klein, 1985).

Angiogenesis begins with the degradation of the parent vessel's BM followed by vascular EC migration outside the vessel wall, which results in vascular sprouts. The ECs of these sprouts proliferate, form lumina, and eventually generate new BM and recruit PCs (Folkman and Haudenschild, 1980).

Extracellular proteolysis is believed to be an essential component of the angiogenic process. Various elements of the angiogenic process may be mediated by extracellular proteolysis including degradation of the basement membrane of the parent vessel and invasion of the interstitial extracellular matrix by migrating ECs. Plasminogen activators (PAs) are key mediators of these processes and PA activity must be balanced by physiological inhibitors such as EC-derived Plasminogen activator inhibitor-1 (PAI-1) for normal capillary morphogenesis (Moscatelli and Rifkin, 1988; Pepper and Montesano, 1990).
During the stage of proliferative retinopathy, active proliferation of new vessels occurs on the retinal surface (Aiello, 1996). Although these vessels rarely cause visual loss, they are fragile and prone to bleed. As a result vitreous haemorrhage is a major component of visual loss during this period. Proliferating vessels are typically accompanied by a fibrous component containing glial tissue which can contract and may lead to traction on the retina. This traction can distort the retina, induce vitreous haemorrhage, or cause retinal detachment. The fibrovascular component often becomes prevalent as the retinopathy progresses. Fibrovascular proliferation in diabetic retinopathy typically proceeds from the disk along the major temporal vascular arcades to encircle the macula with increasing traction. This arrangement has led to the term *wolf-jaw* fibrovascular proliferation as the scar tissue slowly "bites down" on the macula. In the most severe final stages of diabetic retinopathy, this fibrous tissue creates a complete retinal detachment, contracting the retina into a funnel-shape. Untreated, these complications can lead to severe or total irreversible visual loss. The final stage of diabetic retinopathy is quiescence, which occurs both spontaneously over time or following laser photocoagulation. The final visual status of a person with diabetes is determined by whether this quiescence is reached without destruction of visually critical structures in the eye. Laser panretinal photocoagulation seems to accelerate this progression to quiescence therefore, more commonly resulting in inactive disease before the development of sight-threatening complications.

In severe cases, proliferation of new blood vessels may also occur in the anterior portion of the eye, especially on the iris and the anterior chamber angle. If these vessels obstruct the aqueous fluid outflow facilities of the eye, they can lead to neovascular glaucoma which is a severe sight-threatening disorder (Aiello, 1996).

PC loss is an early event in the pathogenesis of diabetic retinopathy. PCs are known to produce an inhibitor of EC growth which appears to be mediated through secretion of activated transforming growth factor-\(\beta\) (TGF-\(\beta\)). When the contact between ECs and PCs is lost, through PC dropout, ECs are free to proliferate (Orlidge and D’Amore, 1987).

Thickening of the extracellular matrix and alteration in its chemical composition is also probably of extreme importance. In diabetes, the documented changes in chemical composition of the BM include increased nonenzymatic glycosylation of collagen, decreased levels of heparan sulphate proteoglycans, and variable reports on changes in fibronectin and laminin. (Williamson *et al.*, 1988). Such changes might alter cell-cell contact and perhaps
allow cellular invasion and breakdown of the BM, one of the earliest features of angiogenesis.

The development of intraocular neovascularization is often correlated with areas of retinal capillary nonperfusion. During retinal development, the growing vessels are noted to invade areas of nonvascularized retina (Michaelson, 1948). In most of the ischaemic retinopathies, neovascularization of the retina or iris is preceded by increasing retinal capillary nonperfusion (Aiello, 1997). Often the neovascularization itself is located at the borders of perfused and nonperfused retina. Therapies which involve destruction of ischaemic retina such as laser photocoagulation or cryotherapy, often result in regression and quiescence of intraocular neovascularization (Aiello, 1997).

These observations suggest that the development and progression of neovascularization may be mediated by factors whose activity is induced by the onset of retinal ischaemia. As early as 1948, Michaelson used these clinical observations as support for the initial growth factor hypothesis concerning regulation of the developing retinal vasculature. This theory, known as the "Michaelson" hypothesis, was later refined by Ashton (1957). It was postulated that ischaemia of the retina produces a factor or factors capable of stimulating the growth of new vessels. To account for the clinical observations noted above, such a factor should be secreted and freely diffusible (accounting for neovascularization of adjacent retinal tissue or distant neovascularization of the iris), should be mitogenic for ECs (to account for proliferation of vessels), should be induced by retinal hypoxia, should have receptors located on retinal ECs (to permit stimulation of this cell type by the molecule), should be increased during periods of active intraocular neovascularization and diminished when neovascularization becomes quiescent either due to natural progression of the disease or successful therapy.

It has now become widely accepted that growth factor release in retinopathy occurs as a consequence of hypoxia (Pfeiffer and Schatz, 1995). Hypoxia causes profound biochemical changes in cells and multiple systems within cells are effected by hypoxia. It is not yet known in detail how cells sense hypoxia, or how this translates into the secretion of angiogenic molecules. Possible mechanisms of hypoxia-sensing are similar to those of additional oxygen-responsive genes such as erythropoietin (EPO) [Fisher et al, 1992; Goldberg et al., 1994; Minchenko et al., 1994a], endothelin-1, interleukin-1α, ornithine decarboxylase (Goldberg et al., 1994), and glucose transporter (Loike et al., 1994). Erythropoietin has recently been shown to induce angiogenesis (Anagnostou et al., 1994).
Similar to erythropoietin, hypoxia-induced VEGF gene expression depends on a haem-
containing protein (Fisher et al., 1992; Goldberg et al., 1994; Minchenko et al., 1994a). 
Furthermore, induction of VEGF in response to hypoxia is due to transcriptional activation 
(Galis et al., 1994).

1.6 MODULATORS OF THE MICROVASCULAR COMPLICATIONS OF 
DIABETIC RETINOPATHY

1.6.1 INTRODUCTION

Proliferation of retinal blood vessels is one of the most striking features of advanced 
diabetic retinopathy. This feature has led to the conclusion that the normal balance of growth 
factors, which usually serves to keep angiogenesis in check, is disturbed in diabetic 
retinopathy (Sharp, 1995) and in ocular disorders such as ROP, leading to a devastating effect 
in the retina. PDR is one of the few examples of human pathological conditions in which 
abnormal growth of new vessels is one of the primary features. In normal ocular tissue, 
angiogenic homeostasis is controlled by the balance between angiogenic stimulators and 
angiogenic inhibitors

1.6.2 Angiogenic Modulators

Growth factors can be defined as multifunctional signals or mediators which modify 
cell growth or proliferation, alone or in concert, by binding to specific cell surface receptors 
(Sporn et al., 1990). Their biological effects on cells include cell adhesion, migration, 
survival, differentiation, extracellular matrix secretion, protease and protease inhibitor 
release, production of other growth activities, and angiogenesis. (Wiedemann, 1992).

A feature of growth factors is that, in most instances, they act locally within the 
tissues in which they are synthesised. This local action of growth factors has led to the 
concepts of 'paracrine' and 'autocrine' action. Paracrine action occurs when a growth factor is 
secreted by a cell and interacts with responsive cells in the immediate vicinity and autocrine 
action occurs when a growth factor is expressed, and acts upon, the same cells (McKay, 
1993).

One of the earliest candidates for a growth factor involved in retinal 
neovascularization was growth hormone (GH), based on clinical observations (Lundbaek et 
al., 1970), but latterly it has been thought that the retina itself produces the factors 
responsible for new vessel growth (Glaser et al., 1980). However, many years elapsed before 
techniques became available which permitted the in vitro demonstration of cell growth-
promoting activity in soluble extracts of retinal tissue (Glaser et al., 1980). Since then, research in this area has progressed to the point where many growth factors and other cytokines with angiogenesis-modulating effects have been described. Some of these factors are stimulatory and some are inhibitory for one or more of the stages of the angiogenic response; others have no effect or have not been tested for a particular activity.

Numerous investigations, in an attempt to identify the factors involved in the neovascular response, have led to the characterisation of several molecules including insulin-like growth factor (IGF) and basic fibroblast growth factor (bFGF). Both of these molecules have shown some association with intraocular neovascularization (Montesano et al., 1986), however, neither fulfils all of the criteria suggested by the Michaelson hypothesis. Although clearly an endothelial mitogen found in the eye (Klein et al., 1970), bFGF lacks a signal sequence necessary for secretion (Abraham et al., 1986). In addition, bFGF has not been consistently associated with active neovascularization in animal or human studies. IGF is a growth-promoting peptide with multiple biological effects (Le Roith and Roberts, 1993). IGF-1 is capable of inducing neovascularization, however, only at concentrations thought to be 10,000 times higher than the levels found in clinical disease (Grant et al., 1993) Therefore, although both FGF and IGF probably play a role in the overall angiogenic response, each has characteristics making them unlikely to be the primary ocular angiogenic growth factor.

Vascular endothelial growth factor (VEGF) appears to be the most promising as the major angiogenic factor of diabetic retinopathy. Even though other growth factors have been implicated in the process of intraocular vascular proliferation, these factors do not show a consistent increase as would be expected if they played a significant causative role (Hannehan et al., 1991; Meyer-Schwickerath et al., 1993).

The following sections will discuss in detail some of the growth factor families thought to be present in retinal neovascularization

1.6.2.1 The VEGF family

Several members of the VEGF family have been identified. VEGF-A was initially identified (Senger et al., 1983; Connolly et al., 1989; Ferrara et al., 1989; Levy et al., 1989; Plouët et al 1989; Conn et al., 1990). At least six different isoforms of VEGF-A polypeptides of different sizes (121, 145, 165, 183, 189 and 206 amino acid residues) are known to exist. These isoforms have distinct but overlapping functions in angiogenesis due to their differential binding to heparin sulphate (Leung et al., 1989; Houck et al., 1991; Tisher et al.,
1991; Claffey et al., 1992; Ferrara et al., 2003; Tammela et al., 2005). Since the discovery of VEGF-A 5 other family members have been identified. VEGF-B, VEGF-C, VEGF-D, VEGF-E (viral) and VEGF-F (snake) [Lyttle DJ et al., 1994; Joukov et al., 1996; Rak et al., 1995; Schmidt et al., 1997; Achen et al., 1998; Wise et al., 1999; Sheta et al., 2000; Veikkola et al., 2000; Shibuya, 2003; Suto et al., 2005]. Structurally, the VEGFs are related to the PDGF family of growth factors, with intrachain and interchain disulphide bonds between eight cysteine residues in conserved positions (Keck et al., 1989; Leung et al., 1989; Tischer et al., 1989; Claffey et al., 1992). The crystal structure of VEGF-A consists of two monomers that are organized in an anti-parallel fashion to form a dimer, with the receptor-binding sites located at each pole of the dimer.

The VEGF receptors, VEGFR-1, VEGFR-2, AND VEGFR-3 belong to the receptor tyrosine kinase (RTK) gene family (Ullrich and Schlessinger, 1990). Each is a type III RTK containing seven extracellular immunoglobulin-like (IgL) domains (Shibuya et al., 1990; Matthews et al., 1991; Terman et al., 1991). They also consist of a transmembrane domain, a juxtamembrane domain, a kinase domain interrupted by a 69 amino acid residue long insert, and a C-terminal tail (Shibuya et al., 1990; Matthews et al., 1991; Terman et al., 1991; de Vries et al., 1992).

High expression of VEGF and its receptors is tightly controlled and restricted to developing microvessels and angioblasts during angiogenesis and vasculogenesis in developing mouse embryos. (Breier et al., 1992; Peters et al., 1993; Quinn et al., 1993; Yamaguchi et al., 1993).

In adults VEGF and its receptors are expressed in healthy organs with generally non-proliferating vessels such as lung, kidney, adrenal gland, liver, stomach, and heart. (Ladoux and Frelin, 1993a; Olofsson et al., 1996) and in organs undergoing vascular remodelling such as in the female reproductive system. (Philips et al., 1990; Galland et al., 1992; Charnock Jones et al., 1993; Shweiki et al., 1993).

In tumours which contain hypoxic regions, VEGF is produced and secreted by blood vessels and tumour cells in the vicinity of tumours and acts on tumour ECs which express VEGF receptors. (Senger et al., 1986; Weindel et al., 1992; Brown et al., 1993a; Brown et al., 1993b; Shweiki et al., 1993; Weindel et al., 1994; Dvorak et al., 1995; Hatva et al., 1995; Leung et al., 1997; Yancopoulos et al., 2000; Padro et al., 2002; Pallares et al., 2006).

VEGF and its receptors are also increased in other pathological disorders characterised by hypoxia, microvascular permeability and neovascularization such as wound
healing (Peters et al., 1993; Nissen et al., 1998), psoriasis (Detmar et al., 1994), and rheumatoid arthritis (Fava et al., 1994; Koch et al., 1994).

During later stages of development and in adult tissues, VEGFR-3 mRNA becomes restricted to developing lymphatic vessels and high endothelial venules (Fournier et al., 1995; Kaipainen et al., 1995; Lymboissaki et al., 1998).

VEGF has been detected in neuronal cells, cardiac myocytes and cornea which are not directly associated with neovascularization (Breier et al., 1992; Plate et al., 1992; Ladoux and Frelin, 1993a).

VEGF and its receptors initiate signalling pathways that play a pivotal role during embryonic development and pathological angiogenesis (Breier, 2000; Yancopulas et al., 2000; Bates and Harper, 2002; Carmeliet and Storkebaum, 2002; Sun et al., 2003; Crol et al., 2004). Heterozygous mutations inactivating the VEGF gene and the VEGFR-2 and VEGFR-1 genes in mice result in embryonic lethality and dramatic defects in angiogenesis and haematopoiesis (Carmeliet et al., 1996; Ferrara et al., 1996; Dumont et al., 1998).

VEGF activates a broad spectrum of biological responses in endothelial cells, including cell proliferation, migration, survival, differentiation and permeability to macromolecules (Senger et al., 1983; Senger et al., 1990; Ladoux et al., 1993a; Waltenberger et al., 1994; Rahimi et al., 2000; Suarez and Ballmer-Hofer, 2001; Yilmaz et al., 2003; Fu and Shen, 2004).

VEGFR-1 transmits only weak mitogenic signals in ECs, however VEGFR-1 may stimulate biological responses in ECs potentially by heterodimerizing with VEGFR-2 (Kanno et al., 2000; Rahimi et al., 2000; Autiero et al., 2003). VEGFR-1 activation in some non-endothelial cells, such as monocytes, is reported to stimulate cell migration and proliferation (Athanassiades et al., 1998). Recently VEGFR-1 was found to play a critical role in recruiting stem cell-differentiated endothelial cells into newly formed blood vessels (Eriksson et al., 2002; Carmeliet, 2003).

VEGFR-2 binds VEGF-A, VEGF-C, and VEGF-D (Wise et al., 1999; Zachary and Gliki, 2001). VEGFR-2 expression is low in quiescent endothelium (Ortega et al., 1997), however, the VEGFR-2 signalling pathway is crucial in bringing about the effects of VEGFs in actively proliferating tissue, including vasodilatation, endothelial cell migration and proliferation (Dejana, 1996; Esser et al., 1998; Kroll and Waltenberger, 1998; Neufield et al., 1999; Rahimi et al., 1999; Yang et al., 1999; Yancopoulos et al., 2000; Venkiteswaran et al., 2002; Calera et al., 2004).
VEGFR-3 binds VEGF-C and VEGF-D (Cao et al., 1998; Marconcini et al., 1999; Makinen et al., 2001; Stacker et al., 2001). VEGFR-3 is believed to play a critical role in the development of the embryonic vascular system but was originally believed to be restricted postnatally to the ECs of lymphatic vessels and specialized fenestrated capillaries (Dumont et al., 1998; Karkkainen et al., 2000; Karkkainen et al., 2001). However, VEGFR-3 may also play a role in adult vascular endothelium. VEGFR-3 expression has been demonstrated to be associated with vascular endothelial cells in tumours and to play a role in vascular tubular morphogenesis (Valtola et al., 1999; Witmer et al., 2001; Clarijs et al., 2002; Persaud et al., 2004). VEGF-D is mitogenic for endothelial cells and given that VEGF-D can activate VEGFR-3, it could be involved in the regulation and/or differentiation of lymphatic and blood vessel endothelium just like VEGF-C (Jeltsch et al., 1997).

1.6.2.2 The VEGF/VEGFR System and Diabetic Retinopathy

VEGF has been postulated to be one of the major retinal angiogenic factors for the following reasons. First, VEGF is known to promote increased vascular permeability and extravasation of plasma proteins (Keck et al., 1989; Ferrara et al, 1992; Senger et al., 1993) resulting in the formation of an extravascular fibrin gel, a substrate for ECs (Dvorak et al., 1995). Fibrin deposition in diabetic retinas has been demonstrated (Murata et al., 1995).

Second, the production of VEGF is enhanced by ischaemia (Ferrara et al., 1992; Shweiki et al., 1993; Senger et al., 1993; Minchenko et al., 1994b; Dvorak et al., 1995) and ocular pathological angiogenesis is almost always associated with the occurrence of capillary nonperfusion and regions of retinal ischaemia. The expression of VEGF, VEGFR-1 and VEGFR-2 occurs in numerous ocular cell types including RPE cells (Adamis et al., 1993; Aiello et al., 1995; Guerrin et al., 1995; Lutty et al., 1996; Ohno- Schwegesinger et al., 2000; Matsui et al., 2001; Grossniklaus et al., 2002), PCs (Aiello et al., 1995; Lutty et al., 1996; Takagi et al., 1996b; Darland et al., 2003), ganglion cells (Pierce et al., 1995; Dorey et al., 1996; Murata et al., 1996), glial cells (Lutty et al., 1996; Pe'er et al., 1996; Sueishi et al., 1996), choriocapillary endothelium (Lutty et al., 1996; Amin et al., 1997), and microvascular ECs (Murata et al., 1995; Stone 1995; Dorey et al., 1996; Lutty et al., 1996; Pe'er et al., 1996; Sueishi et al., 1996; Amin et al., 1997) and their production is dramatically increased in these cells when oxygen levels decrease (Adamis et al., 1993; Aiello et al., 1995; Shima et al., 1995; Sueishi et al., 1996).

Third, the number of receptors for VEGF in retinal ECs is higher than that of ECs in other tissues such as aorta, and cultures of BRECs can proliferate and migrate upon the addition of recombinant VEGF - two crucial steps of retinal angiogenesis (Simorre-Pinatel et
al., 1994; Enaida et al., 1998; Rajah et al., 2002) and it increases in response to hypoxia (Thieme et al., 1995). The higher level of VEGF receptor expression in retinal endothelial cells suggests that the retinal microcirculation may be more sensitive to the effects of VEGF and this may contribute to the pathogenesis of diabetic retinopathy.

Fourth, VEGF is diffusible, because it has the signal sequence needed for secretion and is water soluble (Ferrara et al., 1992; Senger et al., 1993). Other candidate growth factors such as the FGF's lack a classic signal peptide for secretion from the cell (Abraham et al., 1986; Jaye et al., 1986).

VEGF is angiogenic in vivo and its expression, along with expression of VEGFR-1 and VEGFR-2 is temporally and spatially associated with the onset of ischaemia-induced intraocular neovascularization in several animal models (Miller et al., 1994; Pe'er et al., 1995; Pierce et al., 1995; Stone et al., 1996; Aiello et al., 1997; Luna et al., 1997; Ozaki et al., 1997; Segawa et al., 1998; Suzuma et al., 1998; Aliakacem et al., 2000; Wong et al., 2001; Tolentino et al., 2002). In diabetic rats increased levels of VEGFR-1 and VEGFR-2 were expressed in vascular and non-vascular structures of the inner retinas, indicating that the VEGF/VEGFR system is upregulated in early diabetic retinopathy before neovascularization has occurred (Hammes et al., 1998). Inhibition of VEGF reduces the formation and development of new vessels (Adamis et al., 1994; Aiello et al., 1995; Adamis et al., 1996; Qaum et al., 2001; Tolentino et al., 2002). Increased expression of VEGF in the retina is sufficient to cause retinal and subretinal neovascularization (Okamoto et al., 1997; Tobe et al., 1998; Kinnunen et al., 2006) and signalling through VEGF receptors is necessary for retinal neovascularization (Adamis et al., 1996; Robinson et al., 1996; Tolentino et al., 1996; Ozaki et al., 2000; Ferrara et al., 2003). Elevations of VEGF levels have been reported in the ocular fluid of eyes with active proliferative retinopathy as compared with non-diabetic patients, patients with nonproliferative diabetic retinopathy, and quiescent PDR (Adamis et al., 1994; Aiello et al., 1994; Malecaze et al., 1994; Kosano et al., 1999; Mitamura et al., 2002; Ogata et al., 2002ab; Funatsu et al., 2003; Funatsu et al., 2004). Vitreous fluid containing measurable VEGF stimulated the growth of retinal ECs cells in vitro. This stimulation was inhibited by VEGF-neutralising antibodies (Aiello et al., 1994). Plasma levels of VEGF have also been shown to be higher in PDR patients than in non-diabetic controls (Lip et al., 2000).

In developing retinas VEGF expression has been shown to be temporally and spatially correlated with retinal vasculogenesis. VEGF mRNA and proteins are localised in the developing vessels and the angioblasts, i.e. endothelial precursors and to the nerve fibre and
the GCL where the inner vascular network develops, and also in the INL where the outer vascular network develops (Stone, 1995; Dorey et al., 1996; Murata et al., 1996). Once vasculogenesis becomes inactive, VEGF mRNA expression is markedly decreased.

Immunohistochemical studies of rat and human retina have demonstrated that in diabetic retinas, VEGF immunoreactivity is markedly increased in the vascular endothelium and blood vessels walls and is observed in all layers of the retina (Murata et al., 1995; Lutty et al., 1996; Pe'er et al., 1996; Sueishi et al., 1996; Amin et al., 1997; Kunz Mathews et al., 1997; Ishihama et al., 2001; Famigletti et al., 2003).

In situ hybridisation of sections from whole globes which were enucleated at the time of ongoing neovascularization demonstrated that proliferation of vascular elements in PDR and neovascularization of the retina and/or iris secondary to central retinal vein occlusion, retinal detachment, and intraocular tumours were always accompanied by induction of retinal VEGF expression (Pe'er et al., 1995). In each case, expression of VEGF was induced only in a particular layer of the retina (either the ONL, the INL, or the GCL), matching the zones affected by impaired perfusion.

VEGF, VEGFR-2 and VEGFR-1 have also been detected in samples of neovascular membranes from diabetic patients at vitrectomy (Malecaze et al., 1994; Chen et al., 1997; Armstrong et al., 1998a).

1.6.2.3 The Angiopoietin Family

Four members of the angiopoietin family have been identified. Ang-1 and Ang-2 were initially isolated (Davies et al., 1996; Maisonpierre et al., 1997) followed by Ang-3 and Ang-4, which are probably interspecies homologs (Kim et al., 1999a; Valenzuela et al., 1999). Angiopoietins contain an amino-terminal angiopoietin-specific domain followed by a coiled-coil domain, a linker peptide and a carboxy-terminal fibrinogen homology domain (Davies et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999). The fibrinogen homology domain is responsible for receptor binding, the coiled-coil domain is required for dimerization of angiopioietin monomers, and the short amino-terminal region forms ring-like structures that cluster dimers into variable sized multimers necessary for receptor activation (Procopio et al., 1999; Davies et al., 2003; Cho et al., 2004a). Ang-1, Ang-2 and Ang-4 bind to the receptor tyrosine kinase Tie-2. Tie-2 contains two IgL domains, flanking three EGF-like domains followed by three fibronectin type-III repeats in the extracellular region, a single hydrophobic transmembrane region and a split catalytic domain in the cytoplasmic
region (Partanen et al., 1992; Dumont et al., 1993; Iwama et al., 1993; Sato et al., 1993; Schnurch et al., 1993).

Tie-2 expression appears to be mostly restricted to cells of the vascular system during embryogenesis (Sato et al., 1993; Korhonen et al., 1994). Its expression has also been shown to be localised to the endothelium of new vessels undergoing angiogenesis (Wong et al., 1997). Tie-2 is also expressed in certain primitive haematopoietic stem cells (HSCs) and B lymphocytes but not in other lineage-committed cells.

Tie-2 and Ang-1 are co-expressed in quiescent arteries, veins and capillaries, in a wide range of adult tissues (Suri et al., 1996; Maisonpierre et al., 1997; Tsurumi et al., 1997; Stratmann et al., 1998; Audero et al., 2001; Nourhaghighi et al., 2003). Ang-1 is constitutively secreted by periendothelial cells (PCs/SMC's) in these quiescent vessels and in cultured SMC's (Mandriata et al., 1998: Stratmann et al., 1998; Tanaka et al., 1999; Kim et al., 2000a; Loughna and Sato., 2001). This suggests a role for Tie-2 in the maintenance of quiescent vessels at all levels of the vasculature. Ang-1 mRNA expression has also been reported in neurons (Stratmann et al., 1998; Acker et al., 2001; Audero et al., 2001; Hashimoto et al., 2001).

Ang-2 is found only at sites of vascular remodelling including the ovary, placenta, and uterus and is selectively expressed in ECs and in some instances smooth muscle cells (Maisonpierre et al., 1997; Gale et al., 2002). Ang-2 and Tie-2 expression are abundantly expressed in both tumour cells and in ECS of sprouting microvessels of solid tumours (Stratmann et al., 1998; Bunone et al., 1999; Holash et al., 1999a; Takahama et al., 1999; Tanaka et al., 1999; Brown et al., 2000; Etoh et al., 2001; Yu et al., 2001; Nakayama et al., 2004). In contrast to Ang-2, the expression of Ang-1 is not high in most tumours (Hayes et al., 2000).

Tie-2 and Ang-1 appear to play a major role at later stages of vascular development, i.e., during vascular maturation, maintenance of integrity and remodelling, unlike other angiogenic growth factors, such as VEGF, which function during the earliest stages of vascular development. (Keck et al., 1989; Dumont et al., 1994; Sato et al., 1995; Suri et al., 1996; Witzenbichler et al., 1998). Tie-2 has been shown to recruit peri-endothelial cells (Dumont et al., 1994; Sato et al., 1995; Hanahan, 1997). Ang-1 secretion by periendothelial cells in normal quiescent vessels was shown to stabilise vessels by maintaining contacts between ECs and periendothelial cells (Stratmann et al., 1998; Tanaka et al., 1999). However, more recently Uemura et al., 2002 showed that vessel stabilisation by Ang-1 occurred in the absence of pericyte recruitment.
In Ecs Ang-1 induces tube formation, sprouting, adherence, EC differentiation, and survival (Witzenbichler et al., 1998a; Koblizek et al., 1998; Hayes et al., 1999; Kwak et al., 1999; Papapetropoulos et al., 1999; Kim et al., 2000b; Kwak et al., 2000) but not migration (Witzenbichler et al., 1998; Ferrara et al., 1989). Ang-1 also decreases permeability, changes the distribution and activation of junctional adhesion molecules, and stabilizes cell-cell junctions (Gamble et al., 2000; Kim et al., 2000b; Iizasa et al., 2002; Wang et al., 2004; Baffert et al., 2006). Ang-1 also has anti-inflammatory functions (Gamble et al., 2000; Kim et al., 2001a; 2002b; Joussen et al., 2002a; Pizurki et al., 2003; Lemieux et al., 2005).

Ang-2 has been shown to be a natural antagonist of Ang-1 by competitive binding to Tie-2 without stimulating autophosphorylation of the receptor and is thought to play an earlier role at sites of vessel invasion (Maisonpierre et al., 1997). Overexpression of Ang-2 produces vascular defects similar to those in Ang-1 or Tie-2 deficient mice (Keck et al., 1989; Ferrara et al., 1991; Sato et al., 1995; Suri et al., 1996; Korpelainen et al., 1999). In tumours, Ang-2 expression exceeds Ang-1 expression and is specifically associated with small capillaries with few peri-endothelial support cells (Stratmann et al., 1998; Tanaka et al., 1999). Inhibition of Ang-1 by Ang-2 promotes SMC/PC drop-off leading to loosening of the contacts between endothelial and peri-endothelial cells (Hanahan, 1997) which has been shown to be a requirement for rendering and maintaining ECs accessible to angiogenic inducers such as VEGF (Asahara et al., 1998; Korff et al., 2001; Lobov et al., 2002; Visconti et al., 2002; Oshima et al., 2004; Oshima et al., 2005; Scharpfenecker et al., 2005). Down regulation of Ang-2 in quiescent ECs may permit physical interaction of ECs with peri-endothelial cells, leading to inhibition of endothelial proliferation and maturation of the vessel wall. However, in certain in vitro assays Ang-2 was shown to induce-Tie-2 phosphorylation and MMP-9 expression, migration and sprouting, and tube formation of ECs (Maisonpierre et al., 1997; Kim et al., 2000c; Korff et al., 2001; Teichert-Kuliszweska et al., 2001; Mochizuki et al., 2002; Das et al., 2003). This suggests that Ang-2 can act as an agonist depending on cell type and experimental context. Also, overexpression of Ang-2 in cancer cells in mice promotes growth and vascularization of tumours (Tanaka et al., 1999; Ahmad et al., 2001). In contrast, pharmacological inhibition specific to Ang-2 suppresses angiogenesis and growth of tumours (Oliner et al., 2004) and inhibits neovascularization in the rat cornea (White et al., 2003; Oliner et al., 2004).
1.6.2.4 The Angiopoietin Family and Ocular Neovascularization

Ang-2 has been shown to be upregulated in the retinas of diabetic rats, preceding the onset of pericyte loss suggesting that upregulation of Ang-2 plays a critical role in the loss of pericytes in diabetic retina. (Hammes et al. 2004).

Ang-1 was shown to suppress the development of diabetic retinopathy and reduced both vascular endothelial injury and blood-retinal barrier breakdown (Holash et al., 1999a; b; Joussen et al., 2002a). This is consistent with the findings of Thurston et al., 1999 who showed that addition of Ang-1 was able to prevent diabetes-related vascular leakage and leukocyte adhesion and EC death. These effects were mediated in part via down-regulation of VEGF and VEGF has been shown to regulate vascular permeability through downregulation of the tight junction proteins occludin and zonula occluden 1 (Antonetti et al., 1998; Antonetti et al., 1999). In Ang-1-deficient mice ECs are poorly associated with the underlying matrix and do not properly recruit endothelial supporting cells (Suri et al., 1996). Leukocyte adhesion is thought to trigger the disorganization of the EC zonula adherence and tight junctions (Del Maschio et al., 1996; Bolton et al., 1998). They therefore speculated that Ang-1 not only prevents leakage by stabilization of the endothelial-pericyte interaction, but also via inhibition of leukocyte adhesion. The protective role of Ang-1 in the mature vasculature therefore appears to act at several levels: a maturation signal during the development of vessels; as an anti-inflammatory and anti-permeability agent that prevents leukocyte adherence and damage to the EC layer; and in the maintenance of the blood-retina barrier.

Adult mice with induced expression of Ang-1 ubiquitously, or specifically in the retina, appeared normal and had no identifiable changes in retinal or choroidal blood vessels or in retinal function as assessed by electroretinography (Nambu et al., 2004). This inhibition of ocular neovascularization is interesting because overexpression of Ang-1 in skin stimulates neovascularization. Ang-1 also significantly reduced VEGF-induced retinal vascular permeability.

Ang-2 has been shown to be up-regulated in the retina during development of the deep retinal capillaries by angiogenesis and during pathologic angiogenesis in diabetic retina (Adamis et al., 1996; Hackett et al., 2000; Lim et al., 2005; Patel et al., 2005). In human epiretinal membranes obtained from eyes with ischaemic retinal disorders, substantial upregulation of Ang-2 and tie-2 was found than in those from eyes with non-ischaemic diseases, whereas expression of Ang-1 was consistent in all membranes. Both Ang-1 and Ang-2 promoted tube-forming activity and enhanced the effects of VEGF in cultured BREC
(Takagi et al., 2003). Lim et al., 2005 showed that Ang-2 is raised in diabetes. In the absence of VEGF, Ang-2 induces vessel regression but facilitates EC migration and proliferation in concert with VEGF (Etoh et al., 2002; Lobov et al., 2002; Oshima et al., 2005). Therefore, selective up-regulation of VEGF and Ang-2 may lead to aberrant proliferation of leaky, friable vessels, which is characteristic of diabetic eye disease.

Ang-1 and Ang-2 have also been shown to be co-localised with VEGF in human choroidal neovascular membranes (Otani et al., 1999; Hangai et al., 2001). VEGF was shown to upregulate Ang-1 mRNA and protein levels in RPE cells by increasing mRNA stability. This suggests that VEGF may selectively modulate Ang expression during choroidal neovascularization (CNV).

1.6.2.5 TNF-α

Tumour necrosis factor alpha (TNF-α) was discovered as a serum protein released after systemic treatment of rodents with “bacilli Calmette-Guérin” and lipopolysaccharide (Carswell et al., 1975). It was shown to belong to a large family of structurally related proteins called the “TNF Ligand Superfamily”. The actions of these factors are diverse involving inflammation, apoptosis, cell proliferation and the stimulation of various aspects within the immune system (Pfeiffer, 2003; Goetz et al., 2004).

TNF-α is a 26-kDa transmembrane protein containing a C terminus that is external to the cell and cytoplasmic domain. In mammals, TNF can be released from the membrane by a protease of the metalloproteinase/disintegrin/cysteine-rich family called TACE (TNF-alpha converting enzyme), to produce a 17-kDa soluble protein (Black et al., 1997a,b; Blobel, 1997; Ware, 1998). The overall structure of TNF is described as a ‘β-jellyroll’ in which eight antiparallel β-strands form a sandwich 3D structure (Grass, 1996; Ware et al., 1998; Idriss and Naismith, 2000). The TNF-α gene contains four exons and three introns. The average size of mammalian TNF-α is 234 amino acids (Idriss and Naismith, 2000).

TNF-α is produced mainly from monocyte-macrophages but it has also been detected in astrocytes, microglia, smooth muscle cells, endothelial cells, neutrophils, and fibroblasts. (Frangogiannis et al., 1998; Meldrum, 1998; Meldrum et al., 1998). High levels of TNF-α in fluids and serum have been associated with inflammatory processes such as rheumatoid arthritis, Crohns disease, and multiple sclerosis (Brennan and Feldman, 1996; Rink and Kirchner, 1996). In addition, TNF-α is overexpressed in multiple myeloma cell lines and several reports have been associated with the detection of abnormally high levels of TNF-α protein in the blood of cancer patients with a wide range of tumour types (Partanen et al.,
Within groups of patients with the same tumour type higher levels of TNF-α were correlated with advanced tumour stage, greater paraneoplastic complications and shorter survival time.

Upregulation of TNF-α and TNF-α receptor I mRNA and protein in glaucomatous retina has been demonstrated in the inner retinal layers predominantly in glial cells and ganglion cells and it was suggested that TNF-α-mediated cell death, through binding to TNFR-1, is involved in the neurodegeneration process of glaucoma (Tezel et al., 2001).

The biological responses to TNF-α are mediated by two types of TNF receptors, which can be differentiated by their molecular weight of ~ 55 kDa (TNFR1) and 75 kDa (TNFRII). After binding to its receptors intracellular signal transduction pathways activated by TNF-α can be grouped into three general categories. TNF-mediated apoptotic cell death, which is mediated through a caspase cascade (Leong and Karsan, 2000; Petak and Houghton, 2001), TNF-α induced mitogenic, or TNF-α-induced inflammatory responses. TNF-α acts as an inflammatory factor by promoting leukocyte adhesion to vascular endothelium both in vitro and in vivo. Leukocyte adhesion is mediated by the complex interplay of adhesion receptors on both leukocytes and ECs. TNF-α induces the transcription of the leukocyte adhesion molecules ICAM1, VCAM, E-selectin, IL-6, IL-8, and cyclooxygenase (COX)-2, among others (Herz and Gerard, 1993; Rogers et al., 1996; Ridley et al., 1997; Bergstrom et al., 2000; Dunford et al., 2001; Gustin et al., 2004) by activating transcription factors like Activator Protein one (AP-1) and nuclear factor kappa B (NF-κB) [Leong and Karsan, 2000; Wajant and Scheurich, 2001; Hoenen and Berk, 2002; Paria et al., 2003; Viemann et al., 2004; Trickler et al., 2005; Wang et al., 2006]. Zhou et al., 2004 showed that TNF-α activates NFκB, PI3K, and MAPK with concomitant downstream expression of Bcl-2. Bcl-2 is an anti-apoptotic factor whose expression is regulated in response to hypoxia.

TNF-α has also been shown to alter vascular tone (Hollenberg et al., 1991; Luckman et al., 1998) and increase microvascular permeability in endothelial monolayers (Maruo et al., 1992; Brown and Robbins, 1999; Desai et al., 2002; Sedgwick et al., Vandamme et al., 2003; Kerkar et al., 2006). In addition, TNF-α has also been shown to up-regulate the expression of growth factors such as PDGF, VEGF, and Ang-2 (Kim et al., 2000d; Yang et al., 2003; Wang et al., 2006) and to alter the expression of VEGFR-2 (Stadelmann and Lassmann, 2000).

Although originally identified as an endotoxin-induced protein which causes necrosis of tumours (Carswell et al., 1975), TNF-α has recently been shown to mediate tumour progression by causing the proliferation, invasion and metastasis of tumour cells (Balkwill,
2002). When TNF-α is produced by tumours and tumour-associated macrophages or stromal cells at physiological levels, it promotes tumour growth and additional macrophage recruitment, stimulating the release of angiogenic and growth factors from infiltrating cells.

TNF-α has also been shown to modulate neuronal functions (Cunningham et al., 1996; Pan et al., 1997), and to stimulate glial proliferation (Barna et al., 1990; Selmaj et al., 1990; Dopp et al., 1997), and glial activation (Aloisi et al., 1992; Merrill, 1992; Panek et al., 1994; Romero et al., 1996; Munoz-Fernandez and Fresno, 1998).

1.6.2.6 TNF-α and Ocular Angiogenesis

TNF-α overexpression in diabetes is thought to contribute to several complications in diabetes, including retinopathy, nephropathy, neuropathy, and diabetes-enhanced periodontal disease (Nishimura et al., 2003; Satoh et al., 2003; Siragy et al., 2003; Gonzalez-Clemente et al. 2005; Krady et al., 2005). This may result from the effects of hyperglycaemia and advanced glycation end-products (AGES) as has been shown in HUVECs (Rashid et al., 2004). Diabetic retinopathy has an underlying inflammatory component, involving leukocyte recruitment and adhesion to the retinal vasculature and up-regulation of inflammatory genes (Jousson, 2002). Jousson, 2002 showed that leukocyte adhesion in diabetic rat retinas was elevated two-fold more than levels in non-diabetic animals and treatment with the TNF-α receptor inhibitor etenacetept reduced leukocyte adhesion in retinal vessels of treated vs. diabetic controls. Retinal neovascularization was also shown to be abrogated by other selective TNF-α blockers (Zhu et al., 2006).

TNF-α has also been shown to be the predominant pro-inflammatory cytokine observed within the extracellular matrix and luminal and abluminal surface of infiltrating vessels in PDR membranes and has also been demonstrated in PVR membranes (Limb et al., 1996; Armstrong et al., 1998a). Increased levels of TNF-α and its receptors TNFR-I and II have also been detected in the vitreous of eyes with PVR, rhegmatogenous retinal detachment (RRD), and PDR compared with control eyes (Limb, 2001).

Retinal ischaemia has been shown to upregulate TNF, TNFR1 and TNFR2 (Fontaine, 2002; Lahat et al., 2003). TNF-α mRNA was increased in mouse models of retinal neovascularization. (Majka, 2002; Yoshida et al., 2004) and was shown to be expressed by Müller glial cells, in the inner nuclear layer and outer nuclear layer. Yoshida et al., 2004 showed that the TNF-α expression level was enhanced in macrophages/microglia 4 days after hypoxia.
Zhao et al., 2006 showed that TNF-α upregulated VEGF-C and VEGFR-2 expression in retinal ECs. Flow cytometry results showed that VEGF-C prevented EC apoptosis induced by TNF-α and that the anti-apoptotic effect was mainly via VEGFR-2.

TNF-α has also been shown to colocalize with Ang-1, Ang-2 and VEGF in human CNV specimens (Hangai et al., 2006). It was shown to induce the sequential upregulation of Ang-2 and then Ang-1 and VEGF mRNA and protein expression in choroidal microvascular ECs in vitro.

TNF-α has also been shown to produce an angiogenic response in mouse cornea assays which was dramatically increased when Ang-1 or Ang-2 were added suggesting that Tie-2 signalling synergistically amplifies and participates in TNF-α-mediated angiogenesis (Nakoa et al., 2003; Chen et al., 2004).

1.6.3 Inhibitors of Angiogenesis

Numerous inhibitors of angiogenesis have been reported to counteract the effects of growth factors and endogenous angiogenic inhibitors are believed to be essential for maintaining the homeostasis of angiogenesis in the retina (Raymond, 1982). Extracts of cornea and vitreous contain substances that inhibit the proliferation and angiogenesis of endothelial cells.

1.6.3.1 PEDF

Among various molecules with antiangiogenic properties which have been reported to counteract the effects of growth factors, pigment epithelium-derived factor (PEDF) appears to play a prominent role (Mori et al., 2001; Stellmach et al., 2001). Recent studies suggest that the induction of angiogenesis in the eye requires not only an elevation of VEGF but also a decrease in PEDF (Tombran-Tink and Johnson, 1989).

PEDF is a glycoprotein of 50-kDa and a member of the serine protease inhibitors (serpine) superfamily related through their highly conserved folded conformation (Steele et al., 1993; Becerra et al., 1995). PEDF binds to heparin and other glycosaminoglycans in the ECM through lysine residues at a novel binding site for members of the serine family (Simonovic et al., 2001). It was first isolated based on its ability to convert dividing retinoblastoma cells into differentiated neurones, and was therefore characterized as a neurotropic factor (Tombran-Tink and Johnson, 1989; Tombran-Tink et al., 1991). Later, it was shown that besides its neurotropic functions, PEDF is a potent inhibitor of angiogenesis in the eye (Dawson et al., 1999), where it inhibits stimulatory activity of several
proangiogenic factors. Subsequent workers also demonstrated that PEDF is associated with both the cell cycle and senescence (Pignolo et al., 1995; Palmieri et al., 1999; Tresini et al., 1999).

The human PEDF gene spans approximately 16kb and contains eight relatively small exons and seven introns with the largest intron (intron1) approximately 4 kb in length (Tombran-Tink et al., 1996). The human PEDF gene contains an open reading frame encoding a 418-amino-acid protein with a hydrophobic signal that is characteristic of secreted proteins (Steel et al., 1993).

Whether or not PEDF exerts its actions through a classical transmembrane receptor remains an open question. High affinity PEDF-binding sites and proteins have been shown to be present in plasma membranes of the retina, retinoblastoma, CNS, pericytes and ECs, consistent with a cell surface PEDF receptor protein (Wu et al., 1995; Alberdi et al., 1999; Aymerich et al., 2001; Bilak et al., 2002; Filleur et al., 2005). However, little is known about the identity of the receptor and molecular mechanism(s) by which PEDF functions to regulate neuronal and EC behaviour. Notari et al., 2006 found a novel RPE gene, which they termed PEDF-R. They demonstrated the expression of PEDF-R in the retina and showed it had a binding affinity for PEDF.

PEDF mRNA is found in many human foetal and adult tissues and the protein is secreted in primary cultures of various cell types including osteoblasts (Tombran-Tink et al., 1996, 2004; Tombran-Tink and Barnstable, 2004). It is expressed not only in the retina, but also in the vitreous and aqueous humours, in association with fibroblasts, in ciliary epithelium, in cultured RPE, as well as in the adult human brain, the spinal cord, pineal gland, skeletal muscle, bone, heart, placenta liver, teeth, bone and cartilage matrix and human plasma. (Wu et al., 1995; Tombran-Tink et al., 1996; Bilak et al., 1999; Karakousis et al., 2001; Behling et al., 2002; Kunci et al., 2002; Peterson et al., 2003; Tombran-Tink and Barnstable, 2003).

PEDF is among the most potent known natural antiangiogenic factors and it is even more active than angiostatin, thrombospondin-1, and endostatin (Dawson et al., 1999). It plays a role in preventing neovascularization by excluding vessels from invading the retina, vitreous, and cornea (Dawson et al., 1999; Stellmach et al., 2001). PEDF inhibits endothelial cell migration towards many angiogenic factors including platelet-derived growth factor (PDGF), VEGF, IL-8, fibroblast growth factor (FGF), and lysophosphatidic acid (Dawson et al., 1999). PEDF has been shown to inhibit VEGF-induced vascular permeability in mice (Liu et al., 2004) and the inhibitory effect of PEDF on VEGF-induced angiogenesis was
shown to result from the enhanced γ-secretase-dependent cleavage of the C terminus of VEGFR-1, which in turn inhibited VEGFR-2-induced angiogenesis (Cai et al., 2006). In addition, PEDF was also able to regulate the phosphorylation of VEGFR-1, which itself can regulate VEGFR-2 signalling. This identifies two novel pathways by which PEDF inhibits VEGF-induced angiogenesis: regulated intramembrane proteolysis and inhibition of VEGFR-1 phosphorylation.

The antiangiogenic effects of PEDF are associated with induction of endothelial cell apoptosis/cell death (Becerra, 2006). PEDF discriminates between ECs forming new vessels (cells that it destroys) and those that are part of pre-existing vessels (cells that it does not harm) by making use of the same Fas ligand–Fas receptor system that the immune system uses to apoptopically eliminate unwanted lymphocytes (Volpert et al., 2002). In apoptosis, Fas ligand and its receptor, Fas/CD95 are tightly linked to the cell death cascade. When ECs are treated with PEDF Fas ligand expression increases and the Fas/FasL transduction cascade becomes activated which has been shown to lead to cell death of ECs (Volpert et al., 2002). However, it is still unclear whether other pathways are involved in PEDF signalling of apoptotic events since PEDF can still inhibit neovascularization of mice lacking either Fas or FasL (Barreiro et al., 2003). Chen et al., 2006 provide strong evidence that PEDF induces death of ECs through activation of both receptor-mediated and mitochondria-mediated pathways of caspase activation and that activation of these pathways is p38-dependent.

Overexpression of PEDF has been shown to inhibit tumour growth and progression (Crawford et al., 2001; Abe et al., 2004; Matsumoto et al., 2004). The inhibition of tumour growth was shown to occur via PEDF’s anti-proliferative and anti-apoptotic effects and it was shown to suppress VEGF expression in osteoscarcoma cells. Its transcription was shown to decrease with increasing grade of glioma tumour and it was absent in the most aggressive ones (Guan et al., 2003).

PEDF also has neurotrophic, neuronotrophic, neuroprotective and gliastatic properties and acts in neuronal survival and differentiation of photoreceptor and retina cells as well as neurons of the central nervous system (Araki et al., 1994; Cayouette et al., 1999; Cao et al., 2001; Jablonski et al., 2001; Bilak et al., 2002; Kunci et al., 2002).
1.6.3.2 PEDF and Ocular Angiogenesis

Several groups have suggested that shifts in the balance between pro-angiogenic factors such as VEGF and anti-angiogenic factors such as PEDF may be responsible for the pathology seen in choroidal and inner-retinal neovascularization (Gao, 2001; Ogata, 2001a; Ohno-Matsui, 2001; Gao et al., 2002; Gao and Ma, 2002; Ogata, 2002abc). To initiate angiogenesis, the balance between the positive and negative regulators is likely to be shifted such that angiogenic factors are up-regulated or inhibitory factors are decreased.

In foetal and adult human eye tissue, PEDF is expressed by the cornea, ciliary body, the RPE and cells of the inner and outer retina and ganglion cell layer (Karakoisis, 2001; Behling, 2002; Ogata et al., 2002bc; Eichler et al., 2004;). It is thought that PEDF secreted by these cells accumulates in avascular spaces of the eye such as the aqueous and vitreous humour and the interphotoreceptor matrix where it acts as a major inhibitor of angiogenesis (Karakousis, 2001; Behling, 2002).

PEDF is reported to inhibit retinal endothelial cell growth, migration and suppress ischaemia-induced retinal neovascularization (Mori 2001; Stellmach et al., 2001; Duh et al., 2002; Stellmach et al., 2002; Yamagishi et al., 2002, 2003; Duh et al., 2003). In diabetic patients the levels of PEDF has been reported to be lower in the vitreous of patients with PDR (where new vessels are actively forming) than in healthy individuals or in diabetic patients whose vessels are quiescent (Ogata et al., 2001a; 2002a,b,c; Colombo, 2002; Holekamp et al., 2002). Retinal neovascularization, which was induced by hypoxia, was shown to stimulate a reduction in retinal PEDF and increases in VEGF protein and mRNA levels (Gao et al., 2002; Eichler et al., 2004).

AAV-mediated intraocular PEDF gene transfer has been found to increase cell survival after ischaemia-reperfusion injury of the rat retina and cause vessel regression in established neovascularization (Mori, 2001; Stellmach, 2001; Auricchio, 2002; Duh, 2002; Mori et al., 2002ab; Raisler, 2002; Takita, 2003).

PEDF mRNA and protein have shown to be significantly upregulated in RPE cells and the retina after photocoagulation therapy (Ogata, 2001b; Schmidt-Erfurth et al., 2002; Hattenbach et al 2005). Choroidal neovascular tissues, induced experimentally by panretinal laser photocoagulation, contain significant amounts of PEDF mRNA and protein (Ogata et al., 2002c; Martin et al., 2002). The expression of PEDF in these membranes is specifically localized to the proliferating RPE cells that cover the membranes. One reason that may account for the success of panretinal treatment is reducing neovascularization may be, in part, due to an upregulation of PEDF in proliferating RPE cells and its inhibitory effects on
VEGF-induced vessel outgrowth (Ogata et al., 2001b). Matsuoka et al., 2004 showed that PEDF and VEGF were strongly expressed in the vascular ECs and RPE cells in the CNVMs where numerous new vessels were prominent (Clinically active CNVMs). On the other hand, immunoreactivity for PEDF and VEGF was weak in the new vessels where fibrosis was prominent (clinically quiescent CNVMs). However, the RPE cells were still positive for PEDF and VEGF.

It has been suggested that PEDF prevents retinal endothelial cells from responding to ischaemic signals by an apoptotic mechanism (Bouck et al., 2002). Addition of PEDF to the endothelial cultures markedly increased the number of apoptotic cells and apoptotic endothelial cells were more common in retina of hyperoxic animals treated with PEDF than those not receiving PEDF. Mori, 2002c showed that increased expression of PEDF causes regression of ocular neovascularization by promoting apoptosis of cells within neovascular lesions by use of AAV-mediated gene transfer of PEDF in a murine model of choroidal neovascularization. In the cornea, this apoptosis can be shown to be essential because PEDF fails to inhibit angiogenesis as the caspase enzymes required for apoptosis are prevented from functioning (Volpert, 2001).

Zhang et al., 2005 hypothesized that the inhibition of retinal NV and permeability by PEDF is mediated by its anti-inflammatory activity. They showed that retinal and plasma PEDF levels were drastically decreased in rats with endotoxin-induced uveitis (EIU), which suggests that PEDF is a negative acute-phase protein. Intravitreal injection of PEDF significantly reduced vascular hyperpermeability in rat models of diabetes and oxygen-induced retinopathy, correlating with decreased levels of retinal inflammatory factors, including VEGF, VEGFR-2, MCP-1, TNF-α, and ICAM-1. In cultured retinal capillary endothelial cells, PEDF significantly decreased TNF-α and ICAM-1 expression under hypoxia. Moreover, down-regulation of PEDF expression by siRNA resulted in significant increases of VEGF and TNF-α secretion in retinal Müller cells. These findings suggest that PEDF is a novel endogenous anti-inflammatory factor in the eye. The decrease of ocular PEDF levels may contribute to inflammation and vascular leakage in the eye.

Yamagishi et al., 2006 showed that PEDF could inhibit the AGE-induced retinal vascular hyperpermeability and the mechanism by which it might achieve this beneficial effect. AGEs decreased retinal PEDF levels in rats. Treatment with PEDF inhibited the AGE-elicited VEGF-mediated permeability by down-regulating mRNA levels of p22phox and gp91phox, membrane components of NADPH oxidase, and subsequently decreasing retinal levels of an oxidative stress marker. PEDF also inhibited the AGE-induced vascular
hyperpermeability evaluated by transendothelial electrical distance by suppressing VEGF expression. PEDF decreased reactive oxygen species (ROS) generation in AGE-exposed endothelial cells by suppressing NADPH oxidase activity via down-regulation of mRNA levels of p22PHOX and gp91PHOX. This led to blockade of the AGE-elicited RAS activation and NF-κB-dependent VEGF gene induction in ECs. These results indicate that the central mechanism for PEDF inhibition of the AGE signalling to vascular permeability is by suppression of NADPH oxidase-mediated ROS generation and subsequent VEGF expression.

Gao, 2002 showed that intravitreal injection of plasminogen kringle 5 (K5), a potent inhibitor of ischaemia-induced retinal neovascularization (Zhang, 2001) downregulates VEGF, and upregulates PEDF in a dose-dependent manner in cultured retinal vascular cells and in the rat retina. Retinal RNA levels of VEGF and PEDF were also changed by K5.

1.7 THE CAVEOLAE SYSTEM

1.7.1 Introduction

Caveolae (or ‘little caves’) are 50-100 nm plasma membrane invaginations and were originally identified in the 1950’s by electron microscopy (Palade, 1953, Yamada, 1955). They have a very unique lipid composition enriched in cholesterol and sphingolipids (Razani et al., 2002). In many cell types, caveolae occur singularly or in chains or grape-like clusters. (Predescu et al., 1994).

Caveolin (cav), a 21-24 kDa integral membrane protein, is a major protein component of caveolae and is necessary for the formation of invaginated caveolae (Rothberg, 1992; Van Deurs, 2003). Multiple members of the caveolin gene family have been identified (cav-1, cav-2, and -3) that differ in molecular structure and in tissue distribution (Razani et al., 2002). Cav-1 has two isoforms: cav-1α and cav-1β which are translated from a different mRNA. Cav-2 has also been found to have multiple isoforms (α,β,γ) that were discovered in adipocytes (Fra, 1999; Fra, 2000). Among the three caveolins, cav-1 and -3 show high homology (Tang et al., 1996) and either is sufficient for the formation of caveolae invaginations (Fra et al., 1995; Li et al., 1998; Park et al., 2002). Cav-2 requires the presence of cav-1. It has to heterologomerize with cav-1, otherwise it remains trapped within the Golgi complex and is also degraded by cav-1 (Monier, 1995; Sargiocomo, 1995; Scherer, 1997).

Caveolin is composed of cytoplasmic N and C termini and a central intramembrane domain (Carman et al., 1999). The N-terminal region contains the caveolin scaffolding domain, which is essential for both the formation of caveolin oligomers and the interaction of caveolin with a range of other proteins (van Deurs, 2003). The membrane-spanning domain
(MS) forms a hairpin-like loop into the membrane but does not penetrate it. In addition, caveolin is attached to the lipid bilayer by three palmitoyl anchors in the C-terminal region.

Cav-1 null mice are viable but they lack cav-1 protein expression and plasmalemmal caveolae suggesting that cav-1 plays a vital role in caveolae biogenesis (Razani et al., 2001; Razani and Lisanti, 2001). Also analysis of cultured fibroblasts from cav-1 null embryos showed a loss of cav-2 protein expression. Therefore cav-1 expression is required to stabilize the cav-2 protein product.

Caveolae play a role in the internalization and transendothelial trafficking of solutes (Predescu et al., 1994). Caveolar release from the plasma membrane is an important mode of endocytosis in ECs (Predescue et al., 1993), and is the first step in migration of vesicles to the basal membrane (Schnitzer et al., 1996; Oh et al., 1998; Niles et al., 1999; Minshall et al., 2000). The vesicles detach from the plasmalemmal shuttle to the basal membrane where they fuse and release their contents, a process termed transcytosis (Predescu et al., 1994; Ghitescu et al., 1996; Milici et al., 1997; Minshall et al., 2000; Vogel et al., 2001). Caveolae-mediated transcytosis is an important mechanism of transendothelial transport of albumin and delivery of albumin-conjugated nutrients, fatty acids, and hormones across the endothelial barrier (Anderson, 1998).

Caveolin-1 contains a so-called scaffolding domain that is thought to concentrate signalling molecules in caveolae and which binds to and inhibits the activity of several signalling molecules. This allows cav-1 to organize proteins in caveolae through protein-protein interactions, enabling finely tuned regulation of physiological responses (for example, Ca\textsuperscript{2+} entry and eNOS activation) [Drab, 2001].

Many signalling molecules present in caveolae play a role in adhesion, migration, and invasion. They include G-proteins, tyrosine kinases (src, Fyn), receptors for TGF-\beta type II, insulin, IGF-1, EGF, PDGF and VEGF, Angiopoietin, eNOS, and some components of the MAPK pathway. (Li et al., 1995; Liu et al., 1996; Mineo et al., 1996; Liu et al., 1997; Yamamoto et al., 1998; Feng et al., 1999a; Puyraimond 2001; Razani et al, 2002; Huo et al., 2003; Yoon et al., 2003; Schwartz et al., 2005).

A large variety of in vitro and in vivo studies have implicated caveolae and caveolin in the pathogenesis of cancer, atherosclerosis and vasoproliferative diseases, cardiachypertrophy and heart failure, degenerative muscle dystrophies and diabetes mellitus (Schwencke et al., 2006). Several studies have suggested that cav-1 plays a central role in angiogenesis (Liu et al., 1999; Griffoni et al., 2000; Brouet et al., 2001; Woodman et al., 2003; Sonveaux et al., 2004). However, it appears that a bimodal type of regulation seems to
characterise the role of cav-1 in angiogenesis. Cav-1 has been shown to mediate cell transformation, proliferation and capillary tubule formation (Kim et al., 2002b; Griffoni, 2000). Increased expression of cav-1 and microvessel density was shown to correlate with metastasis and poor prognosis in renal cell carcinoma (Joo et al., 2004) Conversely, other workers have shown that Cav-1 inhibits cellular proliferation (Hulit et al., 2000; Razani et al., 2000; Galbiati et al., 2001) and prevents cell transformation (Engelman et al., 1997; Galbiati et al., 1998) and promotes cell-cycle arrest as well as senescence (Galbiati et al., 2001; Volonte et al., 2002). Caveolin-1 has also been shown to function as a tumour suppressor protein in a large variety of cellular settings (Quest et al., 2004; Williams and Lisanti, 2005).

Endothelial-specific overexpression of cav-1 was shown to impair VEGF microvascular permeability and angiogenesis and inhibited the VEGFR-2-mediated angiogenic signalling cascade (Liu et al., 1999). These defects were associated with negative regulation of the PI-3K/Akt/eNOS signalling module, consistent with the established inhibitory action of cav-1 on eNOS (Bucci et al., 2000) and PI-3K activity (Zundel et al., 2000).

Liu et al., 2002 showed that cav-1 is down-regulated by endothelial growth factors (VEGF, PDGF, bFGF, HGF) that stimulate the initial proliferative stage of angiogenesis. This is consistent with the above studies showing that cav-1 is a negative regulator of cell proliferation and cell cycle progression. They also showed that cav-1 expression stimulated EC differentiation and tubule formation.

1.7.2 Systemic Expression of Caveolae and Caveolins

Caveolae can occur at different surface densities in different cell types and they exist most abundantly in terminally differentiated cells including endothelium, adipocytes, type I pneumocytes, fibroblasts, and smooth muscle cells (Kogo et al., 2006) Caveolae and cav-1 are particularly abundant in ECs and comprise 95% of cell surface vesicles and ~15% of EC volume (Predescu et al., 1993). There is a marked variation in caveolar surface density within a given cellular type, which is best exemplified in the case of the different types of endothelium (Simionescu et al., 1974) or mesothelia (Von Ruhland et al., 2004). In the endothelium, the largest population of caveolae occurs in the continuous type, while their numbers are much lower in the fenestrated to occasional caveolae in the endothelium of the discontinuous type (Simionescu et al., 1974).
Cav-1 and cav-2 are co-expressed in most cell types (van Deurs et al., 2002). Cav-1 and cav-2 are usually co-expressed and assembled into hetero-oligomers in the ER and Golgi complex (Scheiffele et al., 1998) and these hetero-oligomeric complexes eventually reach the plasma membrane for caveolae biogenesis (Scherer et al., 1997; Li et al., 1998; Das et al., 1999; Mora et al., 1999; Parolini et al., 1999; Lahtinen et al., 2003; Sowa et al., 2003). Cav-1 is expressed by ECs and pericytes in brain microvessels and in astrocytes surrounding microvessels where it may be involved in blood-brain barrier functioning and also supports coordinated activities between these cells (Virgintino, 2002).

Cav-3 (Way et al., 1996) is expressed in skeletal muscle and myocardium where it is essential for the formation of caveolae (Hagiwara et al., 2000; Galbiati et al., 2001). It was also shown to be expressed in smooth muscle cells (Song et al., 1996), astrocytes (Camero, 1997; Ikezu, 1998), chondrocytes (Nishiyama et al., 1999; Schwab et al., 1999) and sinus ECs (Uehara et al., 2002).

1.7.3 Expression of Caveolae and Caveolins within the Eye

In the eye caveolae were shown to be associated with retinal ECS (Feng et al., 1999b; Kim et al., 2006) and cav-1 has been localized to the RPE (Bridges, 2001). In addition, ROS-derived detergent-resistant membranes (DRMS), and photoreceptor synaptic ribbons (Kachi 2001; Elliot et al., 2003; Martin et al., 2005) were shown to be enriched in cav-1 where it was suggested that it may participate in phototransduction (Seno et al., 2001). Caveolae, cav-1 and cav-2 have also been demonstrated in the lens (Lo and Zhang, 1989; Lo et al., 1998, Lo et al., 2003; Lin et al., 2003; Ruijoi et al., 2003; Lo et al., 2004; Sexton et al., 2004). It was shown to co-immunoprecipitate with PKCγ and connexins 46 and 50 and was suggested to protect against oxidative stress which can trigger cataractogenesis. Sexton et al., 2004 also suggested that in the lens cav-1 may play possible roles in cholesterol trafficking, cell to cell communication and signal transduction. Cav-3 expression was shown in the papillary sphincter smooth muscle cells but not the ciliary muscle and papillary dilator muscle (Kogo et al., 2006). Cav-1 was detected in the ciliary smooth muscle cells and the papillary sphincter muscle cell, but not in the papillary dilator and ciliary muscle. The localisation of caveolin at the cell membrane of corneal epithelium has previously been reported in the context of wound healing (Amino et al., 1997).

Western blot analysis showed that cav-1 and -2 are present in the rat retina (Kim et al., 2006). Immunochemistry indicated that cav-1 was expressed in the majority of the retinal layers, including the GCL, IPL, OPL, and vascular endothelial cells of the retina. Cav-
2 was primarily immunostained in the vessels, but in a few other elements as well. This was the first demonstration of caveolin differential expression in the retina of rats, and suggests that caveolin plays an important role in signal transduction in glial cells and neuronal cells. Russelakis-Carneiro et al., 2004 demonstrated cav-1 expression in the neuronal cell bodies of the retina and in axons of the optic nerve in mice.

Stitt, 2000 showed that AGES bind to receptors in caveolin-rich membrane fractions and are internalized within caveolae organelles in retinal microvascular ECs. In a human retinal endothelial cell line, ECV 304, exposure to VEGF, and other growth factors resulted in a reduced endothelial cell expression of cav-1 which was mediated by a negative feedback mechanism through the VEGFR-2 receptor and subsequent downstream p44/44 MAP kinase pathway (Liu et al., 1999). It can therefore be proposed that cav-1 protein contributes in a unique manner to the angiogenic process in PDR, i.e. growth factors stimulate relaxation of cav-1’s action as a ‘molecular brake’ allowing growth-factor-induced endothelial cell proliferation.
1.8 AIMS

The hypothesis of this study is that spatial and temporal differences in angiogenic growth factors and associated mediators are critical in the progressive stages that result in diabetic retinopathy. Although it has long been established that VEGF is a primary candidate in the pathology of diabetic retinopathy, limited information is available on the exact cellular location of VEGF, VEGF-C and the VEGF receptors in diabetic retina. Furthermore the role, if any, of the angiopoietins and PEDF in diabetic retinopathy needs to be further investigated. Caveolae play an important role in the compartmentalization of growth factor signalling molecules. Consequently, the following studies were undertaken to define these issues.

1) To examine diabetic retinas and categorize the tissue, by clinical and histological examination, into groups relating to the different stages of diabetic retinopathy

2) To perform immunohistochemistry on non-diabetic retinas, diabetic retinas and fibrovascular membranes for:

   a) VEGF and VEGF-C;
   b) The receptors which bind VEGF and VEGF-C;
   c) Ang-1 and Ang-2;
   d) Tie-2, the receptor for Ang-1 and Ang-2.
   e) TNF-α
   f) PEDF
   g) Caveolins-1, -2, and -3

3) Examine the immunostained retinas and fibrovascular membranes by light microscopy and evaluate the intensity of staining semi-quantitatively.
CHAPTER 2. MATERIALS AND METHODS

2.1 MATERIALS AND SOLUTIONS

2.1.1 Chemical Reagents and Solutions

All chemical reagents and antibodies are listed in appendix I together with their source. The constituents of solutions are provided in appendix II.

2.1.2 Human Tissue

Fifty Nine human eyes enucleated and fixed in 10% Neutral buffered formalin (NBF- see Appendix II) within 12 hr post-mortem, were obtained from the National Disease Research Interchange (NDRI), Philadelphia, USA. All eyes, unless stated, were normal and had no known pathological disease.

2.2 METHODS

2.2.1 Clinical assessment of donor eyes

The anterior segment was removed and examination of the posterior segment was performed by Professor David McLeod, a Consultant Ophthalmologist at the Manchester Royal Eye Hospital, using a Zeiss Stemi SV8 zoom dissecting microscope with Schott light source a) to note overt features of retinopathy (e.g. the presence of pre-retinal membranes, cotton wool spots, microaneurysms etc.) and b) to determine the extent of any scatter photocoagulation.

2.2.2 Categorisation of donor eyes

Donor eyes were divided into those with no obvious signs of diabetic abnormalities and those with diabetes. Based on the medical records available and on clinical assessment by stereomicroscopy the diabetic eyes were further divided into four groups. They were categorised as either diabetic with no overt retinopathy, diabetic with intra-retinal changes but no evidence of PDR, diabetic with active proliferative retinopathy or diabetic with scatter laser photocoagulation but no evidence of residual PDR.

2.2.2.1 Non-diabetic eyes

This group contained fourteen human eyes with no known ophthalmic disease, no history of diabetes and no abnormalities on stereomicroscopy. Donors ranged in age from 20 to 92 years (mean 56 years).
2.2.2.2 Diabetic with no overt retinopathy

This group contained twelve human eyes from diabetic donors with no clinical history and no overt macroscopic features of retinopathy or retinal photocoagulation. Donors ranged in age from 44 to 89 years (mean 74 years). A complete medical history was unavailable for all donors but, in those where medical histories were known, the duration of diabetes was between 6 and 25 years.

2.2.2.3 Diabetic with intra-retinal changes but no evidence of PDR

This group contained ten human eyes from diabetic donors with intra-retinal changes on stereomicroscopy but no clinical history and no overt macroscopic features of PDR or retinal photocoagulation. Retinas exhibited cotton wool spots and/or obvious microaneurysms or haemorrhages. Donors ranged in age from 55-96 years (mean 71 years). A complete medical history was unavailable for all donors but, in those where medical histories were known the duration of diabetes was between 3 and 21 years.

2.2.2.4 Diabetic with preretinal PDR

This group contained nine human eyes from diabetic donors defined clinically as having PDR and exhibiting preretinal membranes when examined by stereomicroscopy. All eyes had previously received laser photocoagulation. Donors ranged in age from 37-76 years (mean 58 years). Duration of diabetes ranged from 3-18 years (mean 9 years).

2.2.2.5 Diabetic with scatter laser photocoagulation but no evidence of residual PDR

This group contained fourteen human eyes from diabetic donors defined clinically as having had PDR and having received scatter laser photocoagulation (no details were available as to time post laser). No preretinal membranes could be observed when retinas were examined by stereomicroscopy. Donors ranged in age from 41-82 years (mean 63 years). The duration of diabetes ranged from 3 to 35 years (mean 17 years).

2.2.3 Dissection of donor eyes

The posterior segment of each eye was cut in the sagittal plane through the centre of the optic nerve head. Cuts were then made perpendicular to this line a) on the horizontal midline on the nasal side and b) at approximately 5mm above and below the midline on the temporal side. A final vertical cut was made parallel to the initial cut and approximately 3mm lateral to the macula. For this study tissue was selected from a portion of retina/choroid/sclera
approximately 3mm lateral to the macula and perpendicular to the horizontal plane (this region was chosen owing to its susceptibility to retinal changes associated with diabetes). Tissue was also selected from an area adjacent to the macula region in one eye where a pre-retinal membrane was observed by stereomicroscopy.

2.2.4 Fibrovascular membranes

Seventeen fibrovascular pre-retinal membranes, which had been excised at vitreous surgery from eyes with PDR, were obtained from the Manchester Royal Eye Hospital. Membranes were fixed in 10% NBF immediately upon removal for a minimum of 12 hr before wax embedding.

2.2.5 Preparation of retinal tissue for wax sectioning

Retinal tissue which had been fixed for a minimum of 24 hr in 10% NBF was dehydrated through a graded series of alcohol concentrations (v/v distilled H$_2$O) as follows: 50% alcohol for 30 min, 70% alcohol for 1 hr, 90% alcohol for 1 hr, 2 changes of 100% alcohol for 1 hr each. Dehydrated tissue was then immersed in 50% chloroform (v/v alcohol) for 30 min, 100% chloroform for 1 hr followed by 100% chloroform for 30 min and then embedded in wax for 1.5 hr, using a wax embedding system. The wax blocks were trimmed and 200 serial sections of 7μm thickness were cut on a Microm HM330 microtome. Every 50$^{th}$ section was placed into a warm water bath, allowed to flatten at 47-50°C and then transferred onto microscope slides. The slides were dried in an oven at 56°C overnight to allow the sections to adhere to the slides.

2.2.6 Preparation of fibrovascular membranes for wax sectioning

Fibrovascular membranes which had been fixed for a minimum of 12 hr in 10% NBF were dehydrated through a graded series of alcohol concentrations (v/v distilled H$_2$O) as follows: 25% alcohol for 15 min, 33% alcohol for 15 min, 50% alcohol for 15 min, 63% alcohol for 15 min, 70% alcohol for 30 min, 80% alcohol for 30 min, 90% alcohol for 30 min, 2 changes of 100% alcohol for 30 min each. Dehydrated tissue was then immersed in 25% chloroform (v/v alcohol) for 15 min, 33% chloroform for 15 min, 50% chloroform for 30 min followed by 2 changes of 100% chloroform for 30 min and then immersed in wax for 20 min. The wax blocks were trimmed and serial sections, throughout the whole block, of
5μm thickness were cut. Every 20th section was placed into a warm water bath, allowed to flatten at 47-50°C and then transferred onto microscope slides. The slides were dried in an oven at 56°C overnight to allow the sections to adhere to the slides.

2.2.7 Haematoxylin and eosin staining of wax sections

Sections of retina, and fibrovascular membrane which had been mounted on slides were dewaxed by immersion in two baths of xylene and rehydrated through a graded series of alcohol concentrations (v/v distilled H2O), 5 min in each of 100% alcohol (twice), 90% alcohol, 70% alcohol and 50% alcohol, followed by immersion in cold running water. After excess alcohol had been removed the slides were submerged in a solution of Harris haematoxylin for 3 min then washed in cold running H2O until the dye turned blue. Sections were then submerged in eosin for 1 min, briefly washed in cold running H2O and then dehydrated by immersing them quickly through an alcohol series (the reverse order of that above) followed by two washes with xylene. A coverslip was secured onto each section using Practamount adhesive which was allowed to set by drying overnight in a 60°C oven.

2.2.8 Immunohistochemical studies

2.2.8.1 Selection of sections for immunostaining

Once the sections of retina, and fibrovascular membranes had been stained by haematoxylin and eosin (H and E) they were examined by light microscopy. Non-diabetic retinal sections and diabetic retinal sections showing no overt signs of retinopathy and sections which had overt features of retinopathy, such as pre-retinal membranes, exudates and haemorrhages, were mounted onto 1% Aminopropyltriethoxysilane (APES-see appendix II) coated slides in preparation for immunostaining (sections were selected which were adjacent to the corresponding H and E stained section). Sections of fibrovascular membranes were examined by light microscopy and where blood vessels and a large area of tissue were present adjacent sections were mounted onto 1% Apes coated slides in preparation for immunostaining.
2.2.8.2 General protocol for immunostaining of sections

Sections were dewaxed and rehydrated as previously described (section 2.2.7). Sections were exposed to proteolytic pre-digestion, dependent upon the primary antibody to be used, for between 20 minutes and 30 minutes at 37°C/or in the pressure cooker for 3 minutes (see table 1 and appendix II). Sections were covered for between 20 min and 1 hr at room temperature with the appropriate blocking agent (containing serum from the appropriate secondary antibody host) to block non-specific binding. Sections were washed three times in Tris buffered saline (TBS) [see appendix II]. Primary antibodies were diluted to an appropriate dilution in TBS with 0.2% serum (dilution determined by a dilution series experiment), and added to all the sections, except the negative controls (which received TBS with 0.2% serum) and incubated at 4°C overnight. Slides were washed three times in TBS before addition of the appropriate secondary antibody (biotin conjugated) for 1 hour. After washing three times in TBS, an avidin/biotin complex (ABC) [see appendix II] was applied for 30 min to enhance the antibody signal. After washing the slides three times in TBS a Fast Red substrate or diaminobenzidine (caveolin-1) (see appendix II) for the avidin/biotin complex was filtered and added to the sections to visualise the sites of antibody binding. After washing in running water to quench the substrate reaction slides were counterstained by immersion in a standard solution of Mayers haematoxylin for 10 seconds. Sections were placed in running cold water, air-dried, and coverslips applied using Loctite 358 ultra violet (UV) curing adhesive, which was then sealed by exposure to UV light for 3min.
<table>
<thead>
<tr>
<th>Primary Ab</th>
<th>Host</th>
<th>Pre-treatment</th>
<th>Blocking Agent</th>
<th>Secondary Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF165 (1/20)</td>
<td>Goat</td>
<td>0.1% chymotrypsin</td>
<td>10% Milk proteins/10% Rabbit serum</td>
<td>Rabbit anti-goat biotin conjugate</td>
</tr>
<tr>
<td>VEGF-C (1/100)</td>
<td>Goat</td>
<td>0.1% Chymotrypsin</td>
<td>10% Milk proteins/10% Rabbit serum</td>
<td>Rabbit anti-goat biotin conjugate</td>
</tr>
<tr>
<td>Ang-1 (1/50)</td>
<td>Goat</td>
<td>0.1% Chymotrypsin</td>
<td>Rabbit serum</td>
<td>Rabbit anti-goat biotin conjugate</td>
</tr>
<tr>
<td>Ang-2 (1/50)</td>
<td>Goat</td>
<td>0.1% Chymotrypsin</td>
<td>Rabbit serum</td>
<td>Rabbit anti-goat biotin conjugate</td>
</tr>
<tr>
<td>VEGFR-1 (1/100)</td>
<td>Rabbit</td>
<td>None</td>
<td>10% Milk proteins/10% Goat serum</td>
<td>Goat anti-rabbit biotin conjugate</td>
</tr>
<tr>
<td>VEGFR-2 (1/50)</td>
<td>Rabbit</td>
<td>None</td>
<td>10% Milk proteins/10% Goat serum</td>
<td>Goat anti-rabbit biotin conjugate</td>
</tr>
<tr>
<td>VEGFR-3 (1/100)</td>
<td>Rabbit</td>
<td>None</td>
<td>10% Milk proteins/10% Goat serum</td>
<td>Goat anti-rabbit biotin conjugate</td>
</tr>
<tr>
<td>Tie-2 (1/50)</td>
<td>Rabbit</td>
<td>0.1% Chymotrypsin</td>
<td>Goat serum</td>
<td>Goat anti-rabbit biotin conjugate</td>
</tr>
<tr>
<td>TNF-α (1/25)</td>
<td>Goat</td>
<td>Pressure Cooker</td>
<td>Rabbit serum</td>
<td>Rabbit anti-goat biotin conjugate</td>
</tr>
<tr>
<td>PEDF (1/300)</td>
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<td>Pig serum</td>
<td>Pig anti-rabbit biotin conjugate</td>
</tr>
<tr>
<td>Caveolin-1 (1/10)</td>
<td>Mouse</td>
<td>0.2% Triton X-100</td>
<td>Goat serum</td>
<td>Goat anti-mouse biotin conjugate</td>
</tr>
<tr>
<td>Caveolin-2 (1/10)</td>
<td>Mouse</td>
<td>0.2% Triton X-100</td>
<td>Goat serum</td>
<td>Goat anti-mouse biotin conjugate</td>
</tr>
<tr>
<td>Caveolin-3 (1/10)</td>
<td>Mouse</td>
<td>0.2% Triton X-100</td>
<td>Goat serum</td>
<td>Goat anti-mouse biotin conjugate</td>
</tr>
</tbody>
</table>

Table 2.1 The pre-treatments, blocking agents and secondary antibodies used in the immunostaining for growth factors and their receptors, PEDF, TNF-α, Caveolin-1, -2, and-3
2.2.8.3 Assessment of immunostaining

The degree and pattern of immunostaining both within and between specimens, as observed by standard light microscopy, was assessed by two independent observers (both of which obtained similar results - see appendix V). The intensity of staining was graded qualitatively as background (corresponding to the level of staining seen in the negative controls), weak, moderate, or intense (corresponding to the highest level of immunoreactivity observed). These intensities were recorded as 0, 1, 2, and 3 respectively. For each retinal specimen staining intensity was recorded for photoreceptors, outer retina, inner retina, GCL and retinal vessels. For the fibrovascular membranes staining intensity was recorded for the vessels and the surrounding matrix. An average score was then calculated for each retinal layer within each group.

2.2.8.4 Statistical Analysis

Differences between the retinal layers across tissue categories was assessed using CHI squared statistical analysis (SPSS 12). Values of less than 0.05 were taken to be significant.
CHAPTER 3 HISTOLOGICAL CATEGORIZATION OF NON-DIABETIC AND DIABETIC HUMAN RETINAS AND FIBROVASCULAR MEMBRANES

3.1 INTRODUCTION

59 retinas were stained with haematoxylin and eosin and examined by light microscopy. Each retina was put into a category depending upon whether they were non-diabetic (see table 3.1) or whether they were diabetic and showed any intraretinal microvascular abnormalities associated with diabetic retinopathy (see tables 3.2 to 3.5). The retinas were categorized as either, non-diabetic, unlasered diabetic retinas with no obvious microvascular abnormalities, unlasered diabetic retina with obvious microvascular abnormalities, diabetic retina with PDR or lasered retinas without any obvious microvascular abnormalities. 17 excised fibrovascular membranes were also stained with haematoxylin and eosin to determine if neovessels were absent or present (see table 3.6).

3.2 Haematoxylin and Eosin Staining of Non-diabetic Retinas

None (14/14) of the non-diabetic retinas showed any microvascular changes associated with NPDR or any neovascular membranes associated with PDR (see figure 3.1). None of the retinas examined showed any indication of having previously received photocoagulation treatment. Donor details and the results from haematoxylin and eosin staining are shown below (see table 3.1).
<table>
<thead>
<tr>
<th>Donor Number</th>
<th>Sex</th>
<th>Age</th>
<th>Time Post-Mortem (hours)</th>
<th>Findings On Stereomicroscopy</th>
<th>Findings On H and E</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>79</td>
<td>1</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>88</td>
<td>2</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>75</td>
<td>3</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>82</td>
<td>2</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>63</td>
<td>10.5</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>20</td>
<td>9</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>89</td>
<td>1</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>92</td>
<td>6</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>49</td>
<td>12</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>22</td>
<td>5</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>34</td>
<td>23</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>57</td>
<td>6</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>78</td>
<td>4</td>
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<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>27</td>
<td>8.25</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
</tbody>
</table>
Figure 3.1. H and E Staining of Non-Diabetic Retinas
3.3 Haematoxylin and Eosin Staining of Diabetic Retinas

12/45 of the diabetic retinas examined by light microscopy had no obvious signs of diabetic retinopathy and no indication of having previously received photocoagulation treatment and were placed into the category of unlasered diabetic retinas with no obvious microvascular abnormalities (see table 3.2; figure 3.2). 10/45 of the diabetic retinas examined by light microscopy had microvascular changes associated with NPDR, such as the presence of exudates and gross basement membrane thickening, and were placed into the category of unlasered diabetic retinas with obvious microvascular abnormalities (see table 3.3; figure 3.3). 9/45 of the diabetic retinas had neovascular membranes on their surfaces and were placed into the category of diabetic retinas with PDR (see table 3.4; figure 3.4). 14/45 of the diabetic retinas which had previously had PDR showed evidence of laser treatment but no evidence of NPDR or PDR and were placed into the category of lasered retinas without any obvious microvascular abnormalities (see table 3.5; figure 3.5).
<table>
<thead>
<tr>
<th>Donor Number</th>
<th>Sex</th>
<th>Age</th>
<th>Time Post-Mortem (hours)</th>
<th>Type Of Diabetes</th>
<th>Duration Of Diabetes (years)</th>
<th>Lasered Findings On Stereomicroscopy</th>
<th>Findings On H and E</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>M</td>
<td>77</td>
<td>5</td>
<td>Type 2</td>
<td>9</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>82</td>
<td>4</td>
<td>Type 2</td>
<td>10</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>17</td>
<td>F</td>
<td>89</td>
<td>3</td>
<td>Type 2</td>
<td>6</td>
<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>18</td>
<td>M</td>
<td>57</td>
<td>11</td>
<td>Type 2</td>
<td>5</td>
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<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>19</td>
<td>M</td>
<td>57</td>
<td>11</td>
<td>Type 2</td>
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<td>No microvascular Abnormalities</td>
<td>No microvascular abnormalities</td>
</tr>
<tr>
<td>20</td>
<td>F</td>
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Table 3.3 Unlasered Diabetic Retinas with Obvious Microvascular Abnormalities

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<th>Type Of Diabetes</th>
<th>Duration Of Diabetes (years)</th>
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Table 3.5 Lasered Retinas Without Any Obvious Microvascular Abnormalities

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Figure 3.2. H and E staining of Unlasered Diabetic Retinas with No Obvious Microvascular Abnormalities
Figure 3.3. H and E Staining of Diabetic Retinas With NPDR
Intraretinal haemorrhages (A, B), exudates (C,D) and BM thickening were observed (E,F). Arrows show location of microvascular abnormalities.
Figure 3.4 H and E Staining of Diabetic Retinas With PDR
Arrows show localisation of preretinal membranes
Figure 3.5 H and E Staining of Lasered retinas. Arrows show area of laser burns
3.4 Haematoxylin and Eosin Staining of Fibrovascular Membranes

All (17/17) of the fibrovascular membranes contained blood vessels (see figure 3.6). Donor details are shown below. Medical histories for some donors were not provided (see table 3.6).

Table 3.6 Fibrovascular Membranes

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N/A = No Medical Histories Available
Figure 3.6 H and E Staining of Fibrovascular Membranes
3.5 DISCUSSION

Damage to the retinal microvasculature is a feature of many eye diseases including diabetic retinopathy and some degree of retinopathy occurs in nearly all patients with diabetes of greater than 20 years duration (Klein et al., 1984). Whether retinopathy appears or not depends mainly on the duration of the disease and the degree of metabolic control the patient maintains. Glycaemia-related vascular damage has been hypothesized to be mediated through various biochemical pathways including the hexosamine pathway, the advanced glycation end-product formation pathway, and the diacylglycerol (DAG)-protein kinase C (PKC) pathway (Jawa et al., 2004).

In this study it was important that donors who had previously not been diagnosed with diabetes in their lifetime, be examined for signs of microvascular abnormalities associated with diabetes. This is because it has become increasingly recognized that typical lesions of DR (microaneurysms and haemorrhages) are commonly seen in individuals without clinically diagnosed diabetes (Nguyen and Wong, 2006). However the findings from this study, from stereomicroscopy and from examination of H and E sections, demonstrated that none of the 'non-diabetic' donors showed signs of retinopathy.

I then examined diabetic donors whose medical histories indicated that they had been diagnosed with diabetes but not with DR and whose retinas showed no obvious microvascular abnormalities when viewed by stereomicroscopy. Examination of the H and E sections was consistent with these findings. However, only a small sample of the retina from the macula region was examined by H and E staining as I only stained every 50th section from a total of 200 serial sections. Therefore to get a true picture of what is happening in each retina, it would have been more beneficial to examine every section throughout the macula sample and to take serial sections through all other regions of the retina (the same could apply to the non-diabetic retinas too). Tang et al., 2003 showed that microaneurysms, acellular capillaries and pericyte ghosts were more numerous in the temporal retina than the nasal retina in retinal whole mounts of diabetic patients. However, this does not rule out the presence of microvascular abnormalities in regions other than the macula.

NPDR is characterized by loss of pericytes around capillaries in the retina. This is followed by development of weakness in the capillary wall that leads to capillary aneurysm formation (microaneurysm) and fluid leakage and haemorrhages from capillaries as their walls become more permeable. An increase in the number of microaneurysms is considered a risk sign for progression of retinopathy as well as excessive permeability of the retinal vessels. (Kohner and Sleightholm, 1986; Klein et al., 1995; Ferris et al., 1999). Fluid leakage
can range from microexudates and infiltrating protein or lipid exudates (Davis, 1992; Chew et al., 1996) to the most severe form, macular oedema, which can seriously reduce vision (Ferris and Patz, 1984). Cotton wool spots are also seen which represent stasis of axoplasmic flow due to ischaemia of the nerve fibre layer (Palmberg, 1977; Early Treatment Diabetic Retinopathy Study Research Group, 1991).

In this study I demonstrated that microvascular abnormalities that were present in H and E sections were not always obvious by stereomicroscopy. This emphasises the importance of undertaking H and E staining and the examination of the sections by microscopy. Cotton wool spots and haemorrhages were seen by stereomicroscopy but only basement membrane thickening and exudates could be observed by light microscopy. I also observed haemorrhages with H and E staining.

Histological analysis has also demonstrated the presence of intraretinal haemorrhages, and exudates in several animal models of NPDR (Tolentino et al., 1996; Kim et al., 2004; Kakehashi et al., 2006; Van Eden et al., 2006). Electron, confocal and light microscopy techniques have shown increased BM thickening in numerous diabetic animal models (Fischer et al., 1981; Itabashi et al., 1981; Altschuler and Orney, 1986; Chakrabarti and Sima, 1987; Cuthbertson and Mandel, 1987; Diani et al., 1987; Robinson et al., 1988; Marion and Carlson, 1989; Copeland et al., 1990; Carlson et al., 1997; Miyamura et al., 1999; Hainsworth et al., 2002; Carlson et al., 2003; Gardiner et al., 2003; Joussen et al., 2004; Yatoh et al., 2006). BM thickness was shown to increase at longer duration of diabetes compared with age-matched controls (Feit-Leichman et al., 2005). In addition, ultrastructural comparisons of BMs in a variety of tissues from diabetic and normal human subjects have been carried out (Danowski et al., 1972; Kilo et al., 1972; Dunn et al., 1979; Fischer et al., 1979; Jackson et al., 1982; Johnson et al., 1982; Raskin et al., 1983; Sosenko et al., 1984; Feingold et al., 1986, 1989; Osterby, 1990; Osterby et al., 1998, 2001; Bangstad et al., 1999).

PDR typically develops in patients with type 1 diabetes which I have shown in this study as most of the donors with PDR had type 1 diabetes (Klein et al., 1984a,b; Frank, 2004). In all but 2 of the retinas in this study pre-retinal membranes were observed both by stereomicroscopy and on microscopic examination of H and E sections. It is known that pre-retinal neovascularization may be difficult to detect clinically in the fundus periphery (Tolentino et al., 2002). Therefore the examination of H and E sections was important in this study rather than just viewing the retinas by stereomicroscopy. In rat and mouse models of PDR neovascular membranes were seen in retinas when examined using haematoxylin and eosin staining (Lai et al., 2005; Kakehashi et al., 2006). Tolentino et al., 2002 also
demonstrated the presence of preretinal neovascularization histologically using flat-embedded nonhuman primate retinas.

Studies of histopathology and immunohistochemistry demonstrated that epiretinal membranes (ERMs) consist of complex fibrocellular tissue mainly composed of RPE, macrophages, glial cells, fibroblast-like cells and various amounts of extracellular matrix components and vascular elements (Yamamoto et al., 1989; Hai et al., 1998). I showed that neovessels were present in all the fibrovascular membranes stained by H and E. This is consistent with the findings of Tsanou et al., 2005 who demonstrated that all ERMs surgically removed at vitrectomy had microvessels, with some vessels staining positively for Ki67 which is a marker of proliferation.

Panretinal photocoagulation is the treatment of choice for high-risk retinopathy (The Diabetic Retinopathy Study Research Group, 1979; Whiteside and Thompson, 1989; The Early Treatment Diabetic Retinopathy Study Research Group (ETDRS), 1995). The aim of laser photocoagulation is to eliminate areas of ischaemia, induce the regression of new vessels, and close leaking vessels leading to a decrease in exudates and macula oedema (Petrovic and Bhisitkul, 1999; De La Cruz et al., 2004). Laser treatment of clinically significant macular oedema in patients with diabetic retinopathy is beneficial and reduces the overall risk of visual loss by about 50% (ETDRS No 9, 1991). In some cases, however visual acuity deteriorates (Agardh et al., 1993), which could be due to either a rapid progress of oedema with hard exudates and subretinal fibrosis (ETDRS No23, 1997) or subretinal neovascularization membranes (Lewen, 1988; Varley et al., 1988). However in this study none of the lasered retinas showed signs of exudates or neovascular membranes, when examined by stereomicroscopy and on examination of H and E sections, suggesting that the treatment had been successful.
CHAPTER 4 EXPRESSION OF PRO-ANGIOGENIC GROWTH FACTORS AND THEIR RECEPTORS IN THE NORMAL AND DIABETIC RETINA

4.1 INTRODUCTION

Sections were stained to localise and assess the extent of VEGF-A \(_{165}\), VEGF-C and their receptors VEGFR-1, VEGFR-2 and VEGFR-3 presence in the retina and preretinal membranes. Sections were also stained to localise and assess the extent of angiopoietin-1, angiopoietin-2 and their receptor Tie-2 presence in the retina and preretinal membranes. Finally, sections were stained to localise and assess the extent of TNF alpha presence in the retina and preretinal membranes.

Immunostained sections of retina were examined by light microscopy to determine if there was a temporal and spatial relationship between staining intensity and the various pathological changes associated with diabetic retinopathy.

4.2 CONTROL STAINING

To confirm specificity of the immunostaining control sections were processed with 0.2% non-immune serum in place of the primary antibodies. Staining was negative on all the control sections (see figure 4.1).
Table 4.1 Transverse Sections Showing Negative Control Staining for VEGF-A_{165} (A), VEGF-C (B), VEGFR-1 (C), VEGFR-2 (D), VEGFR-3 (E), Ang-1 (F), Ang-2 (G), Tie-2 (H), and TNF-α (I).
4.3 IMMUNOLOCALISATION OF PRO-ANGIOGENIC GROWTH FACTORS

4.3.1 VEGF-A165, and VEGF-C immunostaining of retinal sections and fibrovascular membranes

When examined by light microscopy, VEGF-A165 staining was apparent in most diabetic tissue but generally absent or weak in the non-diabetic tissue. VEGF-A165 immunoreactivity was generally confined to endothelial cells and perivascular regions. Variability of staining was observed for each retina within each category, which is represented by the standard deviations in table 4.1, but this did not correlate with either donor age, post-mortem time, or duration of diabetes. Statistical analysis demonstrated that significant differences were observed within the retinal vessels across the tissue categories (P = <0.05%) but not within the retinal layers.

The average scores and standard deviations for VEGF-A165 immunostaining are represented in table 4.1. Although staining for VEGF-A165 was generally absent in the non-diabetic retinas, weak staining was associated with the retinal vessels in 7/15 of the specimens. Staining was absent or minimal within the photoreceptors, and the cell bodies in the inner and outer retina and GCL (Fig. 4.2).

In the diabetic retinas with no overt retinopathy VEGF-A165 was absent or minimal within the photoreceptors, and the cell bodies in the inner and outer retina and GCL. Staining was raised in the retinal vessels as compared to the non-diabetic retinas and was observed in 12/19 specimens (Fig. 4.3).

In the diabetic retinas showing vascular changes but no evidence of PDR VEGF-A165 immunostaining was increased within the retinal vessels and the GCL compared to that observed in normal retinas and diabetic retinas with no overt retinopathy. Staining of the photoreceptors, and in the cell bodies in the outer and inner retina was not significantly elevated above that observed in the normal retinas and the diabetic retinas with no overt retinopathy (Fig. 4.4).

Moderate to intense staining of the retinal vessels for VEGF-A165 was observed in all the diabetic retinas (6/6) with active neovascular PDR membranes on their surfaces. Intense staining was also observed within the membranes (Fig. 4.5). Staining was absent within the photoreceptors, within cell bodies in the outer retina, inner retina, and the GCL.

VEGF-A165 immunostaining in diabetic retinas that had undergone apparently successful laser therapy (that is, those with no preretinal neovascularization) showed a
similar staining pattern to that observed for non-diabetic retina (Fig. 4.6). Minimal immunoreactivity was observed within the inner retinal vessels. Staining was absent within the photoreceptors, and the cell bodies of the outer retina, and inner retina. Weak staining was also observed within the GCL.

VEGF-A immunoreactivity was moderate to intense within the preretinal vessels of the excised membranes. Weak to moderate staining was observed within the non-vascular components of most (8/11) of the membranes (Fig. 4.7).

The average scores and standard deviations for VEGF-C immunostaining are represented in table 4.2. When examined by light microscopy, VEGF-C staining was apparent in most non-diabetic and diabetic vascular tissue which was confined to retinal endothelial cells and the perivascular region. Increased immunostaining was observed within intra-retinal vessels of diabetic tissue as compared to non-diabetic tissue. Variable staining of the vessels within each retina was observed with some staining positive and some staining negative. Staining was associated with extravascular regions of the retina. Variability of staining was observed for each retina within each category, which is represented by the standard deviations in table 4.2, but this did not correlate with either donor age, time post mortem, or duration of diabetes. Statistical analysis demonstrated that significant differences were observed within the GCL across the tissue categories (P = <0.05%) but not within the other retinal layers and the retinal vessels.

In the non-diabetic retinas staining intensity for VEGF-C was generally absent or minimal within the photoreceptors, the cell bodies of the outer retina, the inner retina and the GCL. Weak staining was observed within the retinal vessels of 7/14 non-diabetic retinas (Fig. 4.8).

In the diabetic retinas with no overt retinopathy weak to moderate immunoreactivity for VEGF-C was observed within the GCL (8/12), and the retinal vessels (9/12), which was increased as compared with the non-diabetic retinas. Staining intensity was again absent or minimal within the photoreceptors, and the cell bodies of the outer retina and inner retina (Fig. 4.9).

In the diabetic retinas showing vascular changes but no evidence of PDR moderate to intense immunoreactivity for VEGF-C was demonstrated within the retinal vessels (5/5) which was increased, as compared to the retinas with no overt retinopathy (Fig. 4.10). Staining intensity was again weak to moderate within the GCL and absent or minimal within the photoreceptors and the cell bodies of the outer retina and inner retina.
In the diabetic retinas with active neovascular PDR membranes on their surfaces moderate to intense VEGF-C immunostaining of the retinal vessels was observed in all of the sections (6/6) [Fig. 4.11]. However, only minimal staining was observed within the preretinal vessels (2/6) and weak staining was observed within the extravascular matrix (4/6). Staining intensity was also reduced to minimal levels in the GCL (2/6) as compared to the previous categories of diabetic retinas. Staining intensity was minimal within the photoreceptors, and the cell bodies of the outer retina and inner retina.

In those diabetic retinas which had undergone successful laser therapy, staining intensity for VEGF-C was weak within the retinal vessels. Staining was absent or minimal within the photoreceptors, the cell bodies of the outer retina, the inner retina and the GCL (Fig. 4.12).

VEGF-C immunoreactivity was minimal within the preretinal vessels of the excised membranes. Weak staining was observed within the non-vascular components of most (8/11) of the membranes (Fig. 4.13).
TABLE 4.1. MEAN INTENSITY OF VEGF-A165 IMMUNOSTAINING

<table>
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<th>Tissue Category</th>
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<tr>
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<td>Photo-receptors</td>
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<td>Inner Retina</td>
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<td>No Overt Retinopathy (n=12)</td>
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<td>0 (0)</td>
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<tr>
<td>Intraretinal Changes (n=10)</td>
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<td>PDR (n=9)</td>
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<td>0 (0)</td>
</tr>
<tr>
<td>Laser-No Residual PDR (n=14)</td>
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<td>0 (0)</td>
</tr>
<tr>
<td>Excised Membranes (n=17)</td>
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</tbody>
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GCL = ganglion cell layer
0 = background staining
1 = weak staining
2 = moderate staining
3 = intense staining
Values in parenthesis = +/- standard deviation
TABLE 4.2 MEAN INTENSITY OF VEGF-C STAINING

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<td>Vessels</td>
</tr>
<tr>
<td></td>
<td>Photo-receptors</td>
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<td>Inner Retina</td>
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<td>No Overt Retinopathy (n=12)</td>
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<td>0.1 (0.3)</td>
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<td>Intraretinal Changes (n=10)</td>
<td>0.6 (0.9)</td>
<td>0.2 (0.5)</td>
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<tr>
<td>PDR (n=9)</td>
<td>0.3 (0.5)</td>
<td>0.2 (0.4)</td>
<td>0.3 (0.5)</td>
</tr>
<tr>
<td>Laser-No Residual PDR (n=14)</td>
<td>0.4 (0.6)</td>
<td>0.4 (0.9)</td>
<td>0.3 (0.5)</td>
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<td>Excised Membranes (n=17)</td>
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</table>

GCL = ganglion cell layer
ILM = internal limiting membrane

0 = background staining
1 = weak staining
2 = moderate staining
3 = intense staining

Values in parenthesis = +/- standard deviation
Figure 4.2 Transverse Sections Showing the Immunolocalisation (Arrows) of VEGF-A in Non-Diabetic Retinas
Figure 4.3 Transverse Section Showing the Immunolocalisation of VEGF-A (Arrows) in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities.
Figure 4.4 Transverse Sections Showing the Immunolocalisation of VEGF-A (Arrows) in Unlasered Diabetic Retinas with NPDR.
Figure 4.5 Transverse Sections Showing the Immunolocalisation of VEGF-A in Diabetic Retinas with PDR.
Arrows show location of staining in the retinal vessels and the preretinal membranes
Figure 4.6 Transverse Sections Showing the Immunolocalisation of VEGF-A in Lasered Diabetic Retinas
Weak staining was seen in the GCL of some (arrows) of the retinas
Figure 4.7 Transverse Sections Showing the Immunolocalisation of VEGF-A (Arrows) in Fibrovascular Membranes
Figure 4.8 Transverse Sections Showing the Immunolocalisation of VEGF-C (Arrows) in Non-Diabetic Retinas.
Figure 4.9 Transverse Sections Showing the Immunolocalisation of VEGF-C (Arrows) in Unlased Diabetic Retinas with No obvious Microvascular Abnormalities.
Figure 4.10 Transverse Sections Showing the Immunolocalisation of VEGF-C (Arrows) in Unlased Diabetic Retinas with NPDR.
Figure 4.11 Transverse Sections Showing the Immunolocalisation of VEGF-C in Diabetic Retinas with PDR. Arrows show location of staining in the retinal vessels and the preretinal membranes.
Figure 4.12 Transverse Sections Showing the Immunolocalisation of VEGF-C (Arrows) in Lasered Diabetic Retinas.
Figure 4.13 Transverse Sections Showing the Immunolocalisation of VEGF-C (Arrows) in Fibrovascular Membranes
4.3.2 VEGF receptor immunostaining of retinal sections and fibrovascular membranes

When examined by light microscopy immunostaining for all three receptors was observed in both non-diabetic and diabetic vascular and extravascular tissue. The staining pattern depended upon the specificity of the antibody being used and the category of tissue. Increased immunostaining was observed in preretinal and intra-retinal blood vessels of diabetic tissue as compared to non-diabetic tissue. For all the receptors variable staining of the vessels within each retina was observed with some staining positive and some staining negative. In some instances staining was associated with both endothelial cells and the perivascular region of the vessels. Variability of staining was observed for each retina within each category, which is represented by the standard deviations in tables 4.3 to 4.5, but this did not correlate with either donor age, or time post mortem.

The average scores and standard deviations for VEGFR-1 immunostaining are represented in table 4.3. Statistical analysis demonstrated that significant differences were observed within the photoreceptors and the cell bodies of the outer retina across the tissue categories (P = <0.05%) but not within the other retinal layers or the retinal vessels.

In the non-diabetic retinas staining intensity for VEGFR-1 was generally minimal or weak within the photoreceptors, and the cell bodies of the outer retina. Weak to moderate staining was observed within the cell bodies of the inner retina (11/14), the GCL (11/14), and the retinal vessels (9/14) [Fig. 4.14].

In all the diabetic retinas with no overt retinopathy weak to moderate immunoreactivity for VEGFR-1 was associated with the cell bodies of the inner retina (12/12), the GCL (11/12), and the retinal vessels (8/12) [Fig. 4.15]. Immunoreactivity was slightly raised as compared to non-diabetic retinas. Staining within the other retinal layers was generally minimal or weak.

In the diabetic retinas showing vascular changes but no evidence of PDR staining for VEGFR-1 was raised, as compared with the non-diabetic and diabetic retinas with no overt retinopathy, within the cell bodies of the outer retina (3/5), the inner retina (5/5), the GCL (5/5), and the retinal vessels (4/5) [Fig. 4.16]. The most intense staining was observed within the GCL. Staining was again minimal or weak within the photoreceptors.

In the diabetic retinas with active neovascular PDR membranes on their surfaces intensity of staining for VEGFR-1 was similar within the cell bodies of the outer retina (4/6), the inner retina (5/6), the GCL (6/6), and within the retinal vessels (5/6) as compared to the retinas with vascular changes but no PDR (Fig. 4.17). The most intense immunoreactivity for VEGFR-1 was observed within the preretinal vessels of the membranes with all of them
demonstrating positive staining (6/6). In this tissue category staining of the intra-retinal vessels was associated both with the membranes and across the retina. Staining was weak within the non-vascular components of the membranes. Staining was absent from photoreceptors.

In those diabetic retinas which had undergone successful laser therapy staining was weak to moderate within the cell bodies of the inner retina (13/14), the outer retina (14/14), the GCL (14/14), and the retinal vessels (14/14). Staining was again generally minimal within the photoreceptors (Fig. 4.18).

VEGFR-1 immunostaining was weak to moderate within the preretinal vessels of 10/11 of the excised membranes but staining tended to be confined to a proportion of the vessels within each membrane with 4/11 of the membranes demonstrating staining both around the vessels and in the adjacent matrix. Weak staining was associated with the non-vascular components of the membranes (Fig. 4.19).

The average scores and standard deviations for VEGFR-2 immunostaining are represented in table 4.4. Statistical analysis demonstrated that significant differences were observed within the inner retina, the GCL, and the retinal vessels across the tissue categories (P = <0.05%) but not within the other retinal layers.

In the non-diabetic retinas and the diabetic retinas with no obvious retinopathy VEGFR-2 immunoreactivity was absent or minimal within all the retinal layers and within the retinal vessels (Fig. 4.20; Fig. 4.21).

In the diabetic retinas showing vascular changes but no evidence of PDR increased staining was observed within the cell bodies of the inner retina (3/5) and the GCL (4/5) compared to the non-diabetic retinas and the diabetic retinas with no overt retinopathy. Staining was particularly apparent within the Müller cell endfeet in the GCL (Fig. 4.22). VEGFR-2 immunoreactivity was again absent or minimal in all the other retinal layers and within the retinal vessels.

In the diabetic retinas with active neovascular membranes on their surfaces VEGFR-2 immunoreactivity was reduced to minimal levels within the cell bodies of the inner retinal layer and the GCL as compared to the diabetic retinas with vascular changes. Staining was absent or minimal within the other retinal layers. In all retinas moderate to intense staining was observed within the retinal vessels (Fig. 4.23). Moderate staining was also observed within the preretinal vessels of most (5/6) of the membranes. In 4/6 diabetic retinas staining of the intra-retinal vessels was associated with the membranes but in 2/4 of these staining
was also observed in vessels across the retina. Minimal staining was associated with the non-vascular components of the membranes.

In those diabetic retinas which had undergone successful laser therapy staining was reduced to minimal levels within the retinal vessels in comparison to the diabetic retinas with PDR membranes on their surfaces (Fig. 4.24). Staining was again absent or minimal within the retinal layers.

VEGFR-2 staining was weak to moderate within the preretinal vessels of 7/11 of the excised membranes but staining tended to be confined to a proportion of the vessels within each membrane with 2/11 of the membranes demonstrating staining both around the vessels and in the adjacent matrix. Minimal staining was associated with the non-vascular components of the membranes (Fig. 4.25).

The average scores and standard deviations for VEGFR-3 immunostaining are represented in table 4.5. Statistical analysis demonstrated that significant differences were observed within the GCL and the retinal vessels across the tissue categories (P = <0.05%) but not within the other retinal layers.

In the non-diabetic retinas VEGFR-3 immunostaining was generally absent or minimal within all the layers and within the retinal vessels (Fig. 4.26).

In the diabetic retinas with no overt retinopathy all the retinas (12/12) demonstrated increased immunoreactivity for VEGFR-3 within the GCL as compared with the non-diabetic retinas, with most (11/12) also demonstrating increased immunoreactivity for VEGFR-3 within the cell bodies of the inner retina. Staining within the retinal vessels was generally weak with 9/12 retinas showing positive immunoreactivity to VEGFR-3. Staining within the other retinal layers was generally absent or minimal (Fig. 4.27).

In the diabetic retinas showing vascular changes but no evidence of PDR the intensity of staining for VEGFR-3 was similar to that observed for the eyes with no overt retinopathy. Again staining was weak to moderate within the cell bodies of the inner retina (4/5) and the GCL (4/5). Staining was particularly apparent within the Müller cell endfeet in the GCL (Fig. 4.28). Staining within the retinal vessels was weak (2/5). Staining within the other retinal layers was absent or minimal.

In the diabetic retinas with active neovascular PDR membranes on their surfaces the intensity of staining for VEGFR-3 was generally absent or minimal within the photoreceptors and the cell bodies of the outer retina. Staining intensity within the cell bodies of the inner retina was reduced to minimal levels as compared to the unlasered diabetic retinas. Staining intensity within the GCL was weak and so was also reduced compared to the unlasered
diabetic retinas. Weak to moderate staining intensity was observed within the retinal vessels with 5/6 showing positive immunoreactivity for VEGFR-3; staining was slightly raised as compared with that observed in the unlasered diabetic retinas with obvious vascular changes. Weak to moderate staining was also observed within the pre-retinal vessels of the membranes with 5/6 showing positive immunoreactivity for VEGFR-3 (Fig. 4.29). In this tissue category staining of the intra-retinal vessels was associated with the membranes in 3/6 retinas but staining in 2/3 of these was also observed in vessels across the retina. Minimal staining was associated with the non-vascular components of the membranes.

In those diabetic retinas which had undergone successful laser therapy staining was minimal or weak and was generally reduced to the levels observed within the non-diabetic retinas (Fig. 4.30).

VEGFR-3 immunostaining was weak to moderate within the pre-retinal vessels of 10/11 of the excised membranes but staining tended to be confined to a proportion of vessels within each membrane with 2/11 of the membranes demonstrating staining both around the vessels and in the adjacent matrix. Minimal staining was associated with the non-vascular components of the membranes (Fig. 4.31).
TABLE 4.3 MEAN INTENSITY OF VEGFR-1 IMMUNOSTAINING

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<td>Inner Retina</td>
</tr>
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<td></td>
<td>receptors</td>
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<td>Non-diabetic (n=14)</td>
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<td>1.2 (0.9)</td>
</tr>
<tr>
<td>No Overt Retinopathy (n=12)</td>
<td>0.4 (0.5)</td>
<td>0.3 (0.5)</td>
<td>1.4 (0.5)</td>
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<tr>
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<td>0.8 (1.2)</td>
<td>1.2 (1.2)</td>
<td>1.6 (0.8)</td>
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<td>PDR (n=9)</td>
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<td>1.0 (0.8)</td>
<td>1.3 (0.7)</td>
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<tr>
<td>Laser-No Residual PDR (n=14)</td>
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<td>Excised Membranes (n=17)</td>
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GCL = ganglion cell layer
ILM = internal limiting membrane

0 = background staining
1 = weak staining
2 = moderate staining
3 = intense staining

Values in parenthesis = +/- standard deviation
Figure 4.14 Transverse Sections Showing the Immunolocalisation of VEGFR-1 (Arrows) in Non-Diabetic Retinas.
Figure 4.15 Transverse Sections Showing the Immunolocalisation of VEGFR-1 (Arrows) in Unlased Diabetic Retinas with No obvious Microvascular Abnormalities.
Figure 4.16 Transverse Sections Showing the Immunolocalisation of VEGFR-1 (Arrows) in Unlasered Diabetic Retinas with NPDR.
Figure 4.17 Transverse Sections Showing the Immunolocalisation of VEGFR-1 in Diabetic Retinas with PDR. Arrows show location of staining in the retinal vessels and the preretinal membranes, and also in retinal layers where positive staining was observed.
Figure 4.18 Transverse Sections Showing the Immunolocalisation of VEGFR-1 (Arrows) in Lasered Diabetic Retinas.
Figure 4.19 Transverse Sections Showing the Immunolocalisation of VEGFR-1 (Arrows) in Fibrovascular Membranes
### TABLE 4.4 MEAN INTENSITY OF VEGFR-2 IMMUNOSTAINING

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GCL = ganglion cell layer  
ILM = internal limiting membrane  
0 = background staining  
1 = weak staining  
2 = moderate staining  
3 = intense staining  

Values in parenthesis = +/- standard deviation
Figure 4.20 Transverse Sections Showing the Immunolocalisation of VEGFR-2 in Non-Diabetic Retinas. Staining was absent or minimal.
Figure 4.21 Transverse sections showing the immunolocalisation of VEGFR-2 in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities. Staining was absent or minimal.
Figure 4.22 Transverse Sections Showing the Immunolocalisation of VEGFR-2 (Arrows) in Unlased Diabetic Retinas with NPDR.
Figure 4.23 Transverse Sections Showing the Immunolocalisation of VEGFR-2 in Diabetic Retinas with PDR.
Arrows show staining around the retinal vessels and in the preretinal membranes.
Figure 4.24 Transverse Sections Showing the Immunolocalisation of VEGFR-2 in Lasered Diabetic Retinas. Staining was absent or minimal.
Figure 4.25 Transverse Sections Showing the Immunolocalisation of VEGFR-2 (Arrows) in Fibrovascular Membranes
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<td>0.9 (0.9)</td>
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<tr>
<td>No Overt Retinopathy (n=12)</td>
<td>0.3 (0.8)</td>
<td>0 (0)</td>
<td>1.5 (0.8)</td>
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<td>1.8 (1.2)</td>
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<td>PDR (n=9)</td>
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<td>0 (0)</td>
<td>0.3 (0.5)</td>
</tr>
<tr>
<td>Laser-No Residual PDR (n=14)</td>
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<td>0.2 (0.4)</td>
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<td>Excised Membranes (n=17)</td>
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GCL = ganglion cell layer  
ILM = internal limiting membrane  

0 = background staining  
1 = weak staining  
2 = moderate staining  
3 = intense staining  

Values in parenthesis = +/- standard deviation
Figure 4.26 Transverse Sections Showing the Immunolocalisation of VEGFR-3 in Non Diabetic Retinas
Figure 4.27 Transverse Sections Showing the Immunolocalisation of VEGFR-3 in Unlased Diabetic Retinas with No obvious Microvascular Abnormalities
Figure 4.28 Transverse Sections Showing the Immunolocalisation of VEGFR-3 in Unlased Diabetic Retinas with NPDR.
Figure 4.29 Transverse Sections Showing the Immunolocalisation of VEGFR-3 in Diabetic Retinas with PDR.
Figure 4.30 Transverse Sections Showing the Immunolocalisation of VEGFR-3 in Lasered Diabetic Retinas.
Figure 4.31 Transverse Sections Showing the Immunolocalisation of VEGFR-3 in Fibrovascular Membranes
4.3 Angiopoietin immunostaining of retinal sections and fibrovascular membranes

When examined by light microscopy, Ang-1 and Ang-2 staining was apparent in both non-diabetic and diabetic tissue. The staining pattern depended upon the specificity of the antibody being used and the category of tissue. Ang-1 immunoreactivity was generally confined to the vessels of the retina but not to the preretinal vessels of the membranes. Ang-2 immunoreactivity was confined to the preretinal vessels undergoing active neovascularization but only appeared to be associated with the retinal vessels of the non-diabetic retinas and diabetic retinas with no overt retinopathy. Variable staining of the vessels within each retina was observed with some staining positive and some staining negative. Ang-2 immunoreactivity was also observed within the GCL. Variability of staining was observed for each retina within each category, which is represented by the standard deviations in tables 4.6 and 4.7, but this did not correlate with donor age, post-mortem time, or duration of diabetes.

The average scores and standard deviations for Ang-1 immunostaining are represented in table 4.6. Statistical analysis demonstrated that significant differences were observed within the retinal vessels across the tissue categories (P = <0.05%) but not within the retinal layers.

In the non-diabetic retinas staining intensity for Ang-1 was generally absent or minimal within the photoreceptors, the cell bodies of the outer retina, the inner retina, and the GCL. Weak to moderate staining was observed within the retinal vessels of 10/14 of the non-diabetic retinas (Fig. 4.32).

In the diabetic retinas with no overt retinopathy Ang-1 staining intensity was reduced to weak levels within the retinal vessels as compared to the non-diabetic retinas. Staining was again absent or minimal within the photoreceptors, the cell bodies of the outer retina, the inner retina and the GCL (Fig. 4.33).

In the diabetic retinas showing vascular changes but no evidence of PDR, Ang-1 immunostaining was increased within the retinal vessels of 4/5 of the retinas, compared to that observed in the diabetic eyes with no overt retinopathy. Staining was again absent or minimal within the photoreceptors, and the cell bodies of the outer retina, inner retina, and GCL (Fig. 4.34).

In the diabetic retinas with active neovascular PDR membranes on their surfaces staining was increased to moderate levels within the retinal vessels (5/6) compared to the retinas with intraretinal changes. Minimal staining was observed within the preretinal vessels of the membranes and no staining was apparent within the non-vascular components of the
membranes. Staining was absent or minimal within the photoreceptors and the cell bodies of the outer retina, the inner retina, and the GCL.

In those diabetic retinas which had undergone successful laser therapy the staining intensity was reduced to weak to moderate levels within the retinal vessels. Staining was again absent or minimal within the photoreceptors, the cell bodies of the inner retina, the outer retina, and the GCL (Fig. 4.35).

Minimal staining was observed within the preretinal vessels of the excised membranes. Weak to moderate staining was associated with the non vascular components of most (9/11) of the membranes (Fig. 4.37).

The average scores and standard deviations for Ang-2 staining are represented in table 4.7. Statistical analysis demonstrated that no significant differences were observed within the retinal layers and the retinal vessels across the tissue categories.

In the non-diabetic retinas weak to moderate staining was observed within the GCL (11/14) and the retinal vessels (10/14). Minimal staining was demonstrated within the photoreceptors (6/14), and the cell bodies of the outer retina and the inner retina (Fig. 4.38).

In the diabetic retinas with no overt retinopathy, Ang-2 immunostaining was again weak to moderate within GCL (10/12), and the retinal vessels (8/12). Minimal staining was demonstrated within the photoreceptors and the cell bodies of the outer and inner retina (Fig. 4.39).

In the diabetic retinas showing vascular changes but no evidence of PDR, Ang-2 staining was reduced to minimal levels within the retinal vessels (2/5) as compared to the non-diabetic retinas and the retinas with no overt retinopathy. Ang-2 staining intensity was weak to moderate within the GCL (4/5). Minimal staining was demonstrated within the photoreceptors and the cell bodies of the outer retina and inner retina (Fig. 4.40).

In the diabetic retinas with active neovascular PDR membranes on their surfaces 4/6 retinas demonstrated increased immunoreactivity for Ang-2 within the cell bodies of the inner retina as compared with the other categories of tissue. Ang-2 staining intensity was again weak to moderate within the GCL (5/6). Minimal staining was demonstrated within the photoreceptors, the cell bodies of the outer and inner retina and the retinal vessels. The staining intensity was weak to moderate within the preretinal vessels of the membranes. In this tissue category staining of the intraretinal vessels was associated both with the membranes and across the retina. (Fig. 4.41).

In those diabetic retinas which had undergone successful laser therapy Ang-2 staining was again weak to moderate within the GCL (14/14). Minimal staining was
demonstrated within the photoreceptors, the cell bodies of the outer retina, and the inner retina, and the retinal vessels (Fig. 4.42).

Minimal staining was observed within the preretinal vessels and the non-vascular components of the membranes (Fig. 4.43).
TABLE 4.6 MEAN INTENSITY OF ANG-1 IMMUNOSTAINING

<table>
<thead>
<tr>
<th>Tissue Category</th>
<th>Retinal Layer</th>
<th>Retinal Vessels</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Photo-receptors</td>
<td>Outer Retina</td>
<td>Inner Retina</td>
</tr>
<tr>
<td>Non-diabetic (n=14)</td>
<td>0.5 (0.8)</td>
<td>0.1 (0.3)</td>
<td>0.1 (0.4)</td>
</tr>
<tr>
<td>No Overt Retinopathy (n=12)</td>
<td>0.3 (0.4)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Intraretinal Changes (n=10)</td>
<td>0.4 (0.9)</td>
<td>0 (0)</td>
<td>0.2 (0.5)</td>
</tr>
<tr>
<td>PDR (n=9)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.2 (0.4)</td>
</tr>
<tr>
<td>Laser-No Residual PDR (n=14)</td>
<td>0.1 (0.3)</td>
<td>0 (0)</td>
<td>0.1 (0.3)</td>
</tr>
<tr>
<td>Excised Membranes (n=17)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

GCL = ganglion cell layer
ILM = internal limiting membrane
0 = background staining
1 = weak staining
2 = moderate staining
3 = intense staining
Values in parenthesis = +/- standard deviation
Figure 4.32 Transverse Sections Showing the Immunolocalisation of Ang-1 (Arrows) in Non-Diabetic Retinas.
Figure 4.33 Transverse Sections Showing the Immunolocalisation of Ang-1 (Arrows) in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities.
Figure 4.34 Transverse Sections Showing the Immunolocalisation of Ang-1 (where present) [Arrows] in Unlased Diabetic Retinas with NPDR.
Figure 4.35 Transverse Sections Showing the Immunolocalisation of Ang-1 in Diabetic Retinas with PDR.
Photographs from sections with minimal antibody precipitates present are shown.
Figure 4.36 Transverse Sections Showing the Immunolocalisation of Ang-1 (Arrows) in Lasered Diabetic Retinas
Figure 4.37 Transverse Sections Showing the Immunolocalisation of Ang-1 in Fibrovascular Membranes
TABLE 4.7 MEAN INTENSITY OF ANG-2 IMMUNOSTAINING

<table>
<thead>
<tr>
<th>Tissue Category</th>
<th>Retinal Layer</th>
<th>Retinal Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Photo-receptors</td>
<td>Outer Retina</td>
</tr>
<tr>
<td>Non-diabetic (n=14)</td>
<td>0.6 (0.9)</td>
<td>0.2 (0.6)</td>
</tr>
<tr>
<td>No Overt Retinopathy</td>
<td>0.8 (0.6)</td>
<td>0.3 (0.5)</td>
</tr>
<tr>
<td>Intraretinal Changes</td>
<td>0.6 (0.9)</td>
<td>0.2 (0.5)</td>
</tr>
<tr>
<td>(n=10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDR (n=6)</td>
<td>0.3 (0.5)</td>
<td>0.3 (0.8)</td>
</tr>
<tr>
<td>Laser-No Residual PDR</td>
<td>0.6 (0.7)</td>
<td>0.4 (0.6)</td>
</tr>
<tr>
<td>(n=14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excised Membranes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=17)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

GCL = ganglion cell layer
ILM = internal limiting membrane

0 = background staining
1 = weak staining
2 = moderate staining
3 = intense staining

Values in parenthesis = +/- standard deviation
Figure 4.38 Transverse Sections Showing the Immunolocalisation of Ang-2 (Arrows) in Non-Diabetic Retinas
Figure 4.39 Transverse Sections Showing the Immunolocalisation of Ang-2 (Arrows) in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities
Figure 4.40 Transverse Sections Showing the Immunolocalisation of Ang-2 (Arrows) in Unlasered Diabetic Retinas with NPDR.
Figure 4.41 Transverse Sections Showing the Immunolocalisation of Ang-2 (Arrows) in Diabetic Retinas with PDR.
Figure 4.42 Transverse Sections Showing the Immunolocalisation of Ang-2 (Arrows) in Lasered Diabetic Retinas
Figure 4.43 Transverse Sections Showing the Immunolocalisation of Ang-2 in Fibrovascular Membranes.
Staining was absent or minimal.
### 4.3.4 Tie-2 immunostaining of retinal sections and fibrovascular membranes

The average scores and standard deviations for Tie-2 immunostaining are represented in table 4.8. When examined by light microscopy, Tie-2 staining was apparent in most of the non-diabetic and diabetic vascular tissue which was confined to retinal endothelial cells and the perivascular region. Increased immunostaining was observed within the intraretinal vessels of diabetic tissue as compared to non-diabetic tissue. Variable staining of the vessels within each retina was observed with some staining positive and some staining negative. Staining was also associated with extravascular regions of the retina. Variability of staining was observed for each retina within each category, which is represented by the standard deviations in table 4.8, but this did not correlate with either donor age, post-mortem time, or duration of diabetes. Statistical analysis demonstrated that significant differences were observed within the photoreceptors across all the tissue categories (P = <0.05%) but not within the retinal layers or the retinal vessels.

In the non-diabetic retinas weak staining for Tie-2 was observed within the retinal vessels (5/14). Weak to moderate staining was observed within the photoreceptors (11/14) and the cell bodies of the outer retina (13/14), the inner retina (12/14), and the GCL (13/14) [Fig. 4.44].

In the diabetic retinas with no overt retinopathy weak staining was demonstrated within the retinal vessels (6/12). Moderate staining was observed within the photoreceptors (10/12), and the cell bodies of the outer retina (11/12), and the GCL (11/12). However staining intensity was slightly reduced to weak to moderate levels within the cell bodies of the inner retina as compared to the non-diabetic retinas (11/12) [Fig. 4.45].

In the diabetic retinas showing vascular changes but no evidence of PDR the staining intensity was raised to weak to moderate levels within the retinal vessels (4/5) and to moderate levels within the cell bodies of the inner retina (5/5) as compared to the non-diabetic retinas and the retinas with no overt retinopathy. Staining intensity was reduced to weak to moderate levels within the photoreceptors (3/5) and the cell bodies of the outer retina (3/5) as compared to the non-diabetic retinas and the retinas with no overt retinopathy. Staining within the GCL was intense in all (5/5) of the retinas (Fig. 4.46).

In the diabetic retinas with active neovascular PDR membranes on their surfaces the staining intensity was increased to moderate to intense levels within the photoreceptors (6/6) and the cell bodies of the outer retina (6/6) as compared to the retinas with intraretinal changes. Staining intensity was again moderate within the cell bodies of the inner retina (4/6) and weak to moderate within the retinal vessels (3/6). In this tissue category staining of the
intra-retinal vessels was not specifically associated with the membranes. Weak to moderate staining was observed within the pre-retinal vessels of the membranes with 4/6 showing positive immunoreactivity for Tie-2 (Fig. 4.47). Weak to moderate staining was also associated with the non-vascular components of the membranes (3/6).

In those diabetic retinas which had undergone successful laser therapy the intensity of staining showed a similar pattern to that observed in the PDR retinas. However the level of staining was raised to moderate levels within the retinal vessels (11/14) [Fig. 4.48].

Staining for Tie-2 was minimal within the pre-retinal vessels of the excised fibrovascular membranes (2/11). Weak staining was demonstrated within the non-vascular component of the membranes (8/11) [Fig. 4.49].
TABLE 4.8. MEAN INTENSITY OF TIE-2 IMMUNOSTAINING

<table>
<thead>
<tr>
<th>Tissue Category</th>
<th>Retinal Layer</th>
<th>Retinal Vessels</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Photoreceptors</td>
<td>Outer Retina</td>
<td>Inner Retina</td>
</tr>
<tr>
<td>Non-diabetic (n=14)</td>
<td>1.7 (1.1)</td>
<td>1.7 (1.1)</td>
<td>1.7 (1.1)</td>
</tr>
<tr>
<td>No Overt Retinopathy (n=12)</td>
<td>2.3 (1.2)</td>
<td>2.1 (0.1)</td>
<td>1.7 (1.0)</td>
</tr>
<tr>
<td>Intraretinal Changes (n=10)</td>
<td>1.8 (1.6)</td>
<td>1.4 (1.5)</td>
<td>2.0 (1.0)</td>
</tr>
<tr>
<td>PDR (n=9)</td>
<td>2.8 (0.5)</td>
<td>2.8 (0.5)</td>
<td>2.0 (1.6)</td>
</tr>
<tr>
<td>Laser-No Residual PDR (n=14)</td>
<td>2.5 (0.9)</td>
<td>2.5 (0.8)</td>
<td>2.3 (0.7)</td>
</tr>
<tr>
<td>Excised membranes (n=17)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

GCL = ganglion cell layer
ILM = internal limiting membrane

0 = background staining
1 = weak staining
2 = moderate staining
3 = intense staining

Values in parenthesis = +/- standard deviation
Figure 4.44 Transverse Sections Showing the Immunolocalisation of Tie-2 in Non-Diabetic Retinas.
Staining was observed in the retinal vessels and across all the retinal layers.
Figure 4.45 Transverse Tie-2 in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities.
Staining was observed in the retinal vessels and across all the retinal layers.
Figure 4.46 Transverse Sections Showing the Immunolocalisation of Tie-2 in Unlasered Diabetic Retinas with NPDR. Staining was observed in the retinal vessels and across all the retinal layers.
Figure 4.47 Transverse Sections Showing the Immunolocalisation of Tie-2 in Diabetic Retinas with PDR.
Sections are shown where few Tie-2 antibody precipitates are present. Staining was observed in the retinal vessels and across all the retinal layers.
Figure 4.48 Transverse Sections Showing the Immunolocalisation of Tie-2 in Lasered Diabetic Retinas. Staining was observed in the retinal vessels and across all the retinal layers.
Figure 4.49 Transverse Sections Showing the Immunolocalisation of Tie-2 (Arrows) in Fibrovascular Membranes
4.3.5 TNF alpha immunostaining of retinal sections and fibrovascular membranes

When examined by light microscopy immunostaining for TNF-α was observed in both non-diabetic and diabetic vascular and extravascular tissue. Increased immunostaining was observed in preretinal and intraretinal blood vessels as compared to non-diabetic tissue. Variable staining of the vessels within each retina was observed with some staining positive and some staining negative. In some instances staining was associated with both ECs and the perivascular region of the vessel. Variability of staining was observed for each retina within each category, which is represented by the standard deviations in table 4.9, but this did not correlate with either donor age, or time post mortem.

The average scores and standard deviations for TNF-α immunostaining are represented in table 4.9. Statistical analysis demonstrated that significant differences were observed within the outer retina across the tissue categories (P < 0.05%) but not within the other retinal layers or the retinal vessels.

In the non-diabetic retinas staining intensity for TNF-α was generally minimal or absent within the photoreceptors, the cell bodies of the outer retina and the retinal vessels. Weak to moderate staining was observed within the cell bodies of the inner retina (9/11) and the GCL (9/11) [Fig. 4.50].

In the diabetic retinas with no overt retinopathy immunostaining was again generally minimal or absent within the photoreceptors and the cell bodies of the outer retina. Staining was reduced to minimal or absent in the cell bodies of the inner retina as compared to the non-diabetic retinas. Staining was again weak to moderate within the GCL (6/11). The most intense staining for TNF-α was observed within the retinal vessels with all retinas (11/11) showing positive staining [Fig. 4.51].

In the diabetic retinas showing vascular changes but no evidence of PDR staining for TNF-α was again minimal or absent within the photoreceptors and cell bodies of the outer retina. Staining also remained weak to moderate within the cell bodies of the inner retina (5/10) and the GCL (6/10). Staining was reduced to weak to moderate levels in the retinal vessels as compared with the diabetic retinas with no overt retinopathy (4/10) [Fig. 4.52].

In the diabetic retinas with active neovascular PDR membranes on their surfaces staining for TNF-α was absent within the photoreceptors and cell bodies of the outer retina. Staining was reduced within the cell bodies of the inner retina (3/5) and GCL (3/5) as compared to the retinas with vascular changes but no evidence of PDR. Staining was slightly raised in the retinal vessels (3/5) as compared to the retinas with vascular changes but no
evidence of PDR. Staining was weak to moderate within the preretinal vessels of the membranes (2/5) [Fig. 4.53].

In those retinas which had undergone successful laser therapy staining for TNF-α was absent or minimal within the photoreceptors. Staining was raised within the cell bodies of the outer retina as compared to all the other categories of tissue (7/13). Staining was raised to weak to moderate levels in the GCL as compared to the PDR retinas (10/13). Staining was raised within the retinal vessels (9/13) as compared to the PDR retinas (Fig. 4.54).

Weak staining was observed within the preretinal vessels of the excised membranes (5/13). Staining was absent within the non vascular components of the membranes [Fig. 4.55].
## TABLE 4.9 MEAN INTENSITY OF TNF-α IMMUNOSTAINING

<table>
<thead>
<tr>
<th>Tissue Category</th>
<th>Retinal Layer</th>
<th>Retinal Vessels</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Photo-Receptors</td>
<td>Outer Retina</td>
<td>Inner Retina</td>
</tr>
<tr>
<td>Non-diabetic (n=14)</td>
<td>0.1 (0.3)</td>
<td>0.5 (0.3)</td>
<td>1.1 (0.8)</td>
</tr>
<tr>
<td>No Overt Retinopathy (n=12)</td>
<td>0.6 (0.4)</td>
<td>0.6 (0.4)</td>
<td>0.6 (0.4)</td>
</tr>
<tr>
<td>Intraretinal Changes (n=10)</td>
<td>0.4 (0.3)</td>
<td>0.6 (0.5)</td>
<td>1.2 (1.0)</td>
</tr>
<tr>
<td>PDR (n=9)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.8 (0.4)</td>
</tr>
<tr>
<td>Laser-No Residual PDR (n=14)</td>
<td>0.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Excised Membranes (n=17)</td>
<td>1.0 (0.5)</td>
<td>0 (0)</td>
<td>1.0 (0.5)</td>
</tr>
</tbody>
</table>

GCL = ganglion cell layer  
ILM = internal limiting membrane

0 = background staining  
1 = weak staining  
2 = moderate staining  
3 = intense staining  
Values in parenthesis = +/- standard deviation
Figure 4.50 Transverse Sections Showing the Immunolocalisation of TNF-α (Arrows) in Non-Diabetic Retinas.
Figure 4.51 Transverse Sections Showing the Immunolocalisation of TNF-α (Arrows) in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities.
Figure 4.52 Transverse Sections Showing the Immunolocalisation of TNF-α (Arrows) in Unlased Diabetic Retinas with NPDR.
Figure 4.53 Transverse Sections Showing the Immunolocalisation of TNF-α in Diabetic Retinas with PDR.
Arrows showing the location of staining in most of the membranes.
Figure 4.54 Transverse Sections Showing the Immunolocalisation of TNF-α (Arrows) in Lasered Diabetic Retinas.
Figure 4.55 Transverse Sections Showing the Immunolocalisation of TNF-α (Arrows) in Fibrovascular Membranes
4.4 DISCUSSION

The data presented in this study demonstrate that VEGF, the angiopoietins and TNF-α are localised in diabetic tissue and that they are upregulated in diabetic retinopathy. These observations add support for a role of VEGF and its receptors in diabetic retinopathy and indicate the importance of the angiopoietins, its receptor Tie-2 and TNF-α in pathological angiogenesis.

The VEGF/Receptor Family

Immunostaining for VEGF-A, VEGF-C and the VEGF receptors was performed using a dilution series of the primary antibodies, against a set number of sections, as recommended by the manufacturer. Initially only weak staining was observed. Successful staining for VEGF-A was eventually achieved using a highly concentrated dilution of the primary antibody and by exposing the angiogenic bindings sites with proteolytic digestion using 0.1% chymotrypsin. VEGF-C and the receptor antibodies were used at lower dilutions than VEGF-A and in addition proteolytic digestion was also needed to produce satisfactory staining for VEGF-C. Also some non-specific background staining was observed with all antibodies which was removed by blocking with a solution of 10% milk protein/10% rabbit serum prior to addition of the primary antibody. Deposits were observed when examining the sections stained with VEGF-C. After consultation with the manufacturer the VEGF-C antibody was spun at 25000 rpm at 4°C for 10 mins but this did not remove the deposits. Staining for VEGF-C was retried using both a new primary antibody, a new secondary antibody and new fast red substrate but again the deposits were observed. Photographs were taken of areas showing minimal deposits.

VEGF-A was present, albeit at low levels, within the vessels of non-diabetic retinas and diabetic retinas with no overt retinopathy. Several adult organs and tissues in guinea pig and man have been found to constitutively express VEGF-A mRNA (Ladoux et al., 1993b). Various other workers have shown that VEGF-A is expressed weakly within the retinal vessels of human and animal non-diabetic retinas (Murata et al., 1995; Lutty et al., 1996; Gerhardinger et al., 1998; Spirin et al., 1999; Kim et al., 1999b; Witmer et al., 2002). VEGF-A may be secreted in these adult organs in small amounts that are insufficient to induce angiogenesis but may be necessary for regulating baseline microvascular permeability which is essential for tissue nutrition and waste removal and/or maintaining the differentiated state of blood vessels. (Kevil et al., 1998; Luo et al., 1998; Zebrowski et al., 1999; Bates and Harper., 2002; Fu and Shen, 2004; Shibuya, 2005). Kunz Mathews et al. (1997)
demonstrated that increased VEGF-A immunoreactivity was correlated with increased vascular permeability before morphologic changes occurred in the vasculature. It has been shown that the activity of specific Src family kinases is essential for the VEGF-induced enhancement of vascular permeability through the disruption of the VEGFR-2/cadherin/catenin complex (Eliceiri et al., 1999; Weis et al., 2004). Alternatively VEGF-A may be stored within the cells that synthesise it, perhaps awaiting an emergency situation that requires angiogenesis.

In this study the level of VEGF-A expression was raised considerably within the retinal vessels of the eyes with intraretinal vascular changes. This suggests that VEGF exerts its action(s) during the earlier stages of diabetic retinopathy before proliferation occurs. In animal models of background retinopathy and in studies of human diabetic retinas with pre-proliferative diabetic retinopathy VEGF-A expression within the retinal vessels was also shown to be significantly increased as compared to non-diabetic controls (Lutty et al., 1996; Shima et al., 1996; Amin et al., 1997; Segawa et al., 1997; Hammes et al., 1998; Spirin et al., 1999; Ellis et al., 2000; van Eeden et al., 2006; Kaur et al., 2006) and VEGF was shown to induce diabetic induced early retinal abnormalities such as increased vascular permeability (El-Remessey et al., 2003; Cukiernik et al., 2004). VEGF-A was effective in inducing ICAM-1-mediated retinal leukostasis and BRB breakdown in vivo in diabetic rats, indicating that both are important in the pathogenesis of early diabetic retinopathy. (Joussen et al., 2002b; Ishida et al., 2003).

More direct evidence for VEGF-A as a primary candidate in pre-proliferative retinopathy (and PDR) comes from studies in which VEGF-A administration to animals was shown to be sufficient to produce many of the vascular abnormalities common to background diabetic retinopathy (Tolentino et al., 1996; Tolentino et al., 2002; Witmer et al., 2004; Kinnunen et al., 2006). Studies with VEGF inhibitors have confirmed that VEGF plays a central role in ischaemia-induced vascular permeability and intraocular neovascularization (Campochiaro and Hackett., 2003; Patel et al., 2003).

Factors such as oxidative stress, cyclooxygenase-2, prostaglandin E₂, AGES, and IGF-1, which are correlated with diabetic retinopathy may also serve as the primary stimuli to increase retinal VEGF-A expression in background diabetic retinopathy (Segawa et al., 1997; Ellis et al., 2000; Mamputu and Renier, 2002; El-Remessy et al., 2003; Ayalasomayajula et al., 2004; Roybal et al., 2005; Yokoi, 2005; Sreekumar et al., 2006). AGES and Interleukin-6 have been shown to increase retinal VEGF-A expression from...
Müller cells, RPE cells, ECS, and SMCs and pericytes (Hirata et al., 1997; Lu et al., 1998; Endo et al., 2001; Yamagishi et al., 2002; Li et al., 2006; Yao et al., 2006).

The highest level of VEGF-A expression was observed in the intraretinal vessels and preretinal vessels of subjects with active PDR which further supports a role of VEGF-A in PDR. This is consistent with the findings of other studies where VEGF-A was demonstrated in neovascular membranes (Malecaze et al., 1994; Chen et al., 1997; Schneeberger et al., 1997; Armstrong et al., 1998a; Funatsu et al., 2003; Tsanou et al., 2005). Levels of VEGF-A were also significantly higher in the vitreous and aqueous and plasma from PDR subjects than in non-diabetic subjects, subjects with non-proliferative retinopathy, quiescent retinopathy and those which had received laser therapy (Adamis et al., 1994; Aiello et al., 1994; Hernández et al., 1998; Lin Lip et al., 2000; Endo et al., 2001; Umeda et al., 2001; Hogeboom van Buggenum et al., 2002; Mitamura et al., 2002; Ogata et al., 2002abc; Simó et al., 2002; Funatsa et al., 2003; Funatsu et al., 2004; Lip et al., 2004; Yokoi et al., 2005; Ishizaki et al., 2006).

In situ hybridization and immunohistochemical studies on human retinas have demonstrated that proliferation of vascular elements in PDR and neovascularization of the retina and/or iris secondary to central retinal vein occlusion, retinal detachment, and intraocular tumours were always accompanied by upregulation of VEGF-A mRNA (Murata et al., 1995; Pe’er et al., 1995; Lutty et al., 1996; Pe’er et al., 1996; Sueshi et al., 1996; Amin et al., 1997; Kunz Mathews et al., 1997; Witmer et al., 2002). Similarly in animal models of proliferative retinopathy, including ROP, VEGF-A expression was increased in retinal vessels and pre-retinal growths during the period of retinal hypoxia and remained elevated during the development of neovascularization (Pierce et al., 1995; Dorey et al., 1996; Stone et al., 1996; Robbins et al., 1997; Ozaki et al., 1999; Witmer et al., 2002; Bullard et al., 2003). Ozaki et al. (1999) also demonstrated that increased levels VEGF in ischaemic retina showed a temporal and spatial correlation with increased expression of HIF-1α.

All these findings demonstrate that VEGF-A is expressed in the retina prior to the development of neovascularization, remains elevated until neovascularization develops and then declines as the neovascularization regresses. This demonstrates an especially strong correlation between VEGF-A expression and retinal neovascularization.

In this study the finding that immunostaining for VEGF-A is reduced in diabetic retinas that have no overt preretinal neovascularization following laser therapy is consistent with the findings that vitreous and plasma concentrations of VEGF-A decline after successful
laser therapy (Aiello et al., 1994; Lin Lip et al., 2000). It is also consistent with the finding that the levels of VEGF, VEGFR-2 and VEGFR-1 are reduced in neovascular membranes receiving cryotherapy as compared to membranes containing active proliferating vessels (Armstrong et al., 1998a). Both therapies result in the destruction of a large area of ischaemic retinal tissue presumably resulting in a reduction of VEGF-A, the suppression of neovascularization leading to vessel regression and quiescence.

VEGF-C has been shown to stimulate EC migration, proliferation and chemotaxis and has a strong chemotactic effect on VEGFR-3 producing cells in vitro (Joukov et al., 1996; Witzenbichler et al., 1998b; Kroon et al., 1999; Lohela et al., 2003; Saharinen et al., 2004). VEGF-C also stimulated the release of NO, a potential mediator of VEGF-induced angiogenesis, from ECs and increased vascular permeability in the Miles assay (Witzenbichler et al., 1998b). In a rabbit ischaemic hindlimb model VEGF-C promoted angiogenesis (Witzenbichler et al., 1998b). It was also detected in haematopoietic cells and platelets and in bone marrow samples of acute leukaemia patients (Wartivaara et al., 1998). The investigators suggested that VEGF-C release from activated platelets may have a role in angiogenesis during wound healing, and possibly other pathological conditions, such as atherosclerosis, tumour growth, and metastasis. VEGF-C has also been shown to be associated with the regulation of angiogenesis in the lymphatic vasculature (Kukk et al., 1996; Jussila and Alitalo, 2002; Karkkainen et al., 2004). Although no other investigators have looked at VEGF-C expression in diabetic retinopathy, it may not be unreasonable to say that, from the findings of my study, VEGF-C does appear to play some part in the pathogenesis of diabetic retinopathy. It is expressed in the early stages of diabetic retinopathy which suggests its action could be to induce vascular permeability. It is strongly expressed in PDR retinas which suggests that it could be involved in proliferation and migration of endothelial cells. Its expression in the non-diabetics suggests it may also have a role in the quiescent vasculature.

The observation in this study that VEGFR-2 is greatly elevated in both intra- and preretinal vessels in PDR tissue and minimal in the retinal vessels of non-diabetic retina and the quiescent vessels of lasered diabetic retina with no evidence of PDR is in agreement with the view that VEGFR-2 is involved in PDR. VEGF-A and VEGF-C have both been shown to induce invasion, and tube formation when bound to VEGFR-2 (Tille et al., 2003). It is consistent with the finding that VEGFR-2 is present in neovascular membranes and diabetic retinas and in animal models of ischaemia-induced retinal neovascularization (Malecaze et al., 1994; Chen et al., 1997; Armstrong et al., 1998a; Suzuma et al., 1998; Ishida et al., 2000;
Ishimama et al., 2001; Witmer et al., 2002; Gerber and Ferrara., 2003; Cerdan et al., 2004; Wilkinson-Berka et al., 2006). Blockade of VEGFR-2 receptor signalling was sufficient to completely prevent retinal neovascularization (Ozaki et al., 2000). VEGFR-2 may be associated with integrin-dependent migration of ECs, as it forms a complex with integrin αVβ3 upon binding VEGF (Soldi et al., 1999; Hutchings et al., 2003). An interaction between VEGFR-2 and VE-cadherin, a cell-cell adhesion molecule has also been described.

These findings also correlate with studies where VEGFR-2 levels were elevated in non-ocular pathologies that are characterised by neovascularization (Shweiki et al., 1992; Brown et al., 1993a; Brown et al., 1993b; Fava et al., 1994; Abu-Jawdeh et al., 1996; Guidi et al., 1996; Leung et al., 1997; Samaniego et al., 1998; Hiratsuka et al., 2002; Inoue et al., 2002; Stewart et al., 2003; Takekosh et al., 2004; Pallares et al., 2006), and in embryogenesis where VEGFR-2 expression has been shown to be imperative for EC mitogenesis, the formation of blood vessels and for haematopoiesis. (Ferrara et al., 1996).

Although VEGFR-2 gene expression appears to be upregulated in various pathologies characterised by hypoxia, in vitro studies have yielded conflicting results. The level of VEGFR-2 expression appears either to decline or not change by exposure to acute hypoxia (Brogi et al., 1996; Takagi et al., 1996a) whereas exposure to prolonged periods of hypoxia results in an increase in VEGF binding sites (Thieme et al., 1995). Although the in vivo significance of increased VEGF-A expression, combined with initial decreased VEGFR-2 expression observed in vitro, is not certain, Takagi et al. (1996a) suggested that the physiological significance of the biphasic VEGF receptor response may be to regulate hypoxia induced neovascularization more tightly. Initial, possibly transient, decreases in oxygen concentrations, where VEGF-A levels can be dramatically elevated but where angiogenesis may not be urgently required, lead to a reduction of VEGFR-2 and thus an amelioration of VEGF-A's angiogenic stimuli. However, under conditions of chronic oxygen deficits, in which angiogenesis is a more appropriate response, VEGF receptors are increased and so potentially facilitate VEGF action. This is supported by the finding that hypoxia increases VEGF receptor number by 50% in cultured BRECs (Thieme et al., 1995). In vitro studies have demonstrated that adenosine is a mediator of the angiogenic effects of VEGF-A through the regulation of VEGFR-2 expression during acute hypoxia (Takagi et al., 1996a). Adenosine plays a major role in neuronal and vascular responses of the retina to alterations in oxygen delivery (Ghiardi et al., 1999; Adair 2005).
The presence of VEGFR-1 in non-diabetic tissue adds support to the suggestion that VEGF-A has a function in endothelial maintenance and vascular permeability, for example, and that these effects are mediated through VEGF-A binding to the VEGFR-1 receptor.

The presence of VEGFR-1 in diabetic vessels, particularly in those undergoing active neovascularization, indicates that VEGFR-1 plays a role in both pre-proliferative retinopathy and PDR. As VEGFR-1 has previously been shown to promote vascular permeability (Kolch et al., 1995), in diabetic retinopathy it may be involved in transducing signals within ECs which induce vascular leakage. It is upregulated during hypoxia (Gerber et al., 1997; Partanen et al., 1999. It may also be co-expressed with VEGFR-2 and it has been suggested that it may participate in VEGF-A induced mitogenesis by forming a heterodimer with VEGFR-2 (Waltenberger et al., 1994). VEGFR-1-mediated signalling appears to modulate the reorganization of actin via p38 MAPK, whereas VEGFR-2 contributes to the reorganization of the cytoskeleton by phosphorylating FAK (focal adhesion kinase) and paxillin, suggesting a different contribution of the two receptors to the chemotactic response (Kanno et al., 2000). VEGFR-1 has also been shown to act as a negative regulator of VEGF-A induced angiogenesis; a soluble form of VEGFR-1 can form a VEGF-A-stabilised ternary complex with the extracellular region of VEGFR-2 in vitro (Kendall et al., 1996). In support of this, endogenous trophoblast DNA synthesis was shown to be increased 3-fold in the presence of anti-VEGFR-1 antibody but not in the presence of anti-VEGFR-2 antibody (Ahmed et al., 1997).

VEGFR-1 has previously been detected in samples of neovascular membranes and diabetic retinas (Malecaze et al., 1994; Chen et al., 1997; Armstrong et al., 1998a; Witmer et al., 2002). In addition, these findings also correlate with studies where VEGFR-1 levels were elevated in other pathologies that are characterised by neovascularization (Peters et al., 1993; Fava et al., 1994; Leung et al., 1997; Pallares et al., 2006) and in developing embryos (Fong et al., 1995; Ferrara et al., 1996) indicating that VEGFR-1 is essential for endothelial differentiation, EC and vessel organisation, blood vessel growth, and vascular repair.

The findings from this study that VEGFR-3 is elevated in diabetic vessels, particularly in the intra- and preretinal vessels of the PDR retinas suggest that VEGFR-3 may have a role in the pathogenesis of diabetic retinopathy. As mentioned previously, VEGF-C is a ligand for this receptor which is known to have angiogenic effects on endothelial cells (Joukov et al., 1996; Witzenbichler et al., 1998b). In developing mouse embryos, VEGFR-3 is specifically expressed in endothelial precursors although its expression does become confined to higher venules and the lymphatic system in adults (Kaipainen et al., 1993;
Kaipainen et al., 1994a; Lymboussaki et al., 1998; Partanen et al., 2000; Makinen et al., 2001; Veikkola et al., 2001). Targeted inactivation of the gene encoding VEGFR-3 resulted in defective blood vessel development in early mouse embryos indicating that VEGFR-3 is important for the remodelling and maturation of primary vascular networks into larger blood vessels (Dumont et al., 1998). VEGFR-3 has also been detected in samples from patients with myeloid leukaemia, and in various tumour cell lines including a retinoblastoma cell line, indicating it plays a role in pathological neovascularization (Pajusola et al., 1992; Fielder et al., 1997; Partanen et al., 1999; Valtola et al., 1999; Skobe et al., 2001ab; Witmer et al., 2001; Clarijs et al., 2002). A role for VEGFR-3 in adult angiogenesis was shown by Witmer et al., 2004 who demonstrated that VEGFR-3 was expressed in pre-existing blood vessels in human tissues undergoing angiogenesis and in a VEGF-A induced model of iris neovascularization. VEGFR-3 has also been demonstrated in retinal vessels during early diabetic retinopathy (Witmer et al., 2002). VEGFR-3 has recently been shown to heterodimerize with VEGFR-2 in ECs and stimulates VEGFR-2 signalling in response to VEGF-C (Alam et al., 2004; Suzuki et al., 2005). Together VEGFR-3 and VEGFR-2 induced the formation of capillary-like structures and the proliferation of human ECs. Use of an anti-human VEGFR-3 monoclonal antibody that antagonized the receptor activation by VEGF-C resulted in the reduction of tubule formation (Persaud et al., 2004).

Various workers have demonstrated that production of VEGF and its receptors is not specifically confined to retinal vascular ECs in the diabetic retina. VEGF has been shown to be highly expressed in ganglion cells and glial cells (Müller cells and astrocytes) in non-diabetic retina (Famigietti et al., 2003) and diabetic retina (Pe'er et al., 1996; Sueshi et al., 1996; Amin et al., 1997; Hammes et al., 1998). In addition VEGF expression is also significantly raised in these cells in animal models of ischaemic retinopathy (Shima et al., 1996; Stone et al., 1996; Robbins et al., 1997; Kaur et al., 2006) and during the development of the retinal vasculature (Stone et al., 1995). RGCs have been shown to synthesise and release VEGF which is enhanced by hypoxia (Sueshi et al., 1996; Jingjing et al., 1999) and conditioned media from hypoxic RGCs stimulates in vitro angiogenesis in collagen gels (Jingjing et al., 1999). Therefore VEGF appears to be released by Müller cells and astrocytes under hypoxic conditions. Increased VEGFR-2 and VEGFR-1 expression have also been demonstrated in the GCL, INL, and ONL in animal models of background retinopathy and ischaemia-induced retinal neovascularization (Hammes et al., 1998; Suzuma et al., 1998; van Eeden et al., 2006) with VEGFR-1 mRNA also being present in cultured retinal glial cells (RGCs), and glial cells of epiretinal membranes. (Chen et al., 1997).
Retinal capillaries are largely ensheathed by perivascular glial cells, which participate in the formation of barrier properties in capillaries (Janzer et al., 1987; Tout et al., 1993). In my study VEGF-A levels were raised in the GCL of the unlasered diabetic retinas with microvascular abnormalities. Therefore it may be reasonable to say that VEGF localised in glial cells plays some role as a vascular permeability factor both in normal retinal vessels and in the early stages of diabetic retinopathy. This VEGF expression may make the retinal vessels permeable so that the retinal cells can get a supply of oxygen and nutrition. This is supported by the finding of Sueshi et al. (1996) who demonstrated that VEGF expression in diabetic retina was associated with vascular hyperpermeability and that astrocytes intimately surrounded these blood vessels.

As glial cells and ganglion cells appear to be a major source of VEGF in ischaemic retina, the above studies suggest that glial cells and possibly ganglion cells are able to detect hypoxia and in response they secrete VEGF and increase their expression of the VEGF receptors. During retinal development and the associated network of blood vessels, astrocytes were shown to be sensitive to hypoxia and astrocytes only enter retinas in which the retinal vasculature will form (Stone et al., 1987; Schnitzer, 1988ab). In response to hypoxia the astrocytes are able to migrate ahead of the developing vessels and secrete VEGF, inducing the formation of developing vessels toward the VEGF-producing astrocytes (Stone et al., 1995). In support of this, astrocytes in avascular retina promote fibronectin production, and fibronectin can provide guidance for migrating spindle cells (EC precursors and glial cells) and extending vessels (Jiang et al., 1994). Astrocytes can also induce ECs to form capillary-like structures in culture (Laterra et al., 1990). As the hypoxic pressures ease after the arrival of the blood vessels, the production of VEGF by astrocytes decreases. In the INL of the developing retina VEGF is also expressed transiently by cells which are presumed to be Müller cells. As Müller cells extend from the ILM to the OLM of the retina, they are thought to be affected by abnormal biologic activities in the vitreous. Penn et al. (1988) showed that the b-wave of electroretinograms, which is produced by Müller cell activity alone, is specifically affected in oxygen-induced rats. This finding points to a significant effect of variable oxygen levels on Müller cell function and suggest that Müller cells are important in building the vasculature, maintaining vascular homeostasis, and promoting neovascularization.

However, VEGF is present in glial cells of retinas from patients without proliferative retinopathy, indicating that hypoxia may not be the sole stimulus for VEGF expression from these cells (Sueshi et al., 1996; Amin et al., 1997; Hammes et al., 1998). The production of
VEGF protein by hypoxic retinal glial cells *in vivo* may be influenced by glucose concentrations (Brooks *et al.*, 1998) and elevation of AGEs in the vitreous may also increase the expression and release of VEGF from Müller cells. Adenosine is also present in the GCL, INL, and the plexiform layers of the retina (Ghiardi *et al.*, 1999), and as it has been shown to regulate VEGFR-2 expression (Takagi *et al.*, 1996a), its release from glial cells and possibly other cell types under hypoxic conditions may be of relevance in the pathogenesis of diabetic retinopathy. Adenosine was also shown to increase VEGF-induced proliferation of canine retinal microvascular cells (Lutty *et al.*, 1998).

Müller cells and astrocytes also share the ability to form the glia limitans of the retina and of vessels (Holländner *et al.*, 1991). In ROP, preretinal vessels form when intensive hypoxia causes the degeneration of astrocytes and the strong expression of VEGF by other cells, particularly neurones (Chan-Ling *et al.*, 1992; Chan-Ling *et al.*, 1995ab; Stone *et al.*, 1996). Therefore the glia limitans becomes damaged, and with high expression of VEGF and the glia limitans breached, VEGF may diffuse in to the vitreous and induce vessel growth away from the retina. PDR may therefore be caused by exaggeration of the mechanisms that cause normal vascularization of the retina; preretinal vessels may from, as do normal vessels, by the hypoxia-induced secretion of VEGF and may be proliferative because of the high level of VEGF expression.

Takagi *et al.* (1996b) demonstrated that bovine retinal PCs (BRPCs) predominantly express VEGFR-1 in contrast to retinal ECs which predominantly express VEGFR-2. Witmer *et al.*, 2004 also demonstrated that activated pericytes express VEGFR-1 in a monkey model of iris neovascularization. Retinal PCs possess large numbers of high affinity VEGF binding sites, which result in tyrosine phosphorylation of intracellular substrates and weak growth promoting effects after long-term VEGF stimulation. This suggests that VEGF may mediate the response of retinal PCs as well as retinal ECs during the pathological angiogenesis characteristic of PDR and other ischaemic retinal disorders.

Hypoxia also increased VEGF expression in BRPCs. Although the role of PCs during angiogenesis is poorly understood, it is likely that hypoxia would turn them predominantly mitogenic, resulting in their loss of contact with endothelial cells, thereby promoting endothelial cell growth. AGES have been shown to upregulate the secretory forms of VEGF mRNA in retinal pericytes (Yamagishi *et al.*, 2002). This suggests that AGES disturb retinal microvascular homeostasis by inducing pericyte apoptosis and VEGF overproduction, therefore playing a role in the pathogenesis of early diabetic retinopathy.
The Angiopoietin/Tie-2 Family

Immunostaining for Ang-1, Ang-2, and Tie-2 was performed using a dilution series of the primary antibodies, against a set number of sections, as recommended by the manufacturer. Initially only weak staining was observed for all antibodies. Successful staining was eventually achieved using a highly concentrated dilution of each primary antibody and by exposing the angiogenic binding sites with proteolytic digestion using 0.1% chymotrypsin. Deposits were observed when examining the sections stained with all three antibodies. After consultation with the manufacturer the antibodies were spun at 25000 rpm at 4°C for 10 mins but this did not remove the deposits. Staining was retried using new primary antibodies, a new secondary antibody and new fast red substrate but again the deposits were observed. Photographs were taken of areas showing minimal deposits. The sections were imaged several months after staining and some drying out of the mountant was observed which is evident from the photomicrographs.

In this study Ang-1 and Tie-2 were localised to the endothelial and perivascular cells of both the non-diabetic and diabetic retinas. In the vessels of the non-diabetic retinas and the diabetic retinas with no overt retinopathy this is consistent with the observation that Ang-1 is expressed in normal arterial and venous specimens (Witzenbichler et al., 1998a) and in BRECs and BAECs (Oh et al., 1999). Tie-2 is also expressed and phosphorylated in the entire spectrum of the quiescent vasculature (arteries, veins and capillaries) which also suggests a role for Tie-2 signalling in the maintenance of the quiescent adult vasculature (Wong et al., 1997). Maisonpierre et al., 1997 also demonstrated that during ovulation Ang-1 is expressed in early follicles where the vasculature is in a quiescent state.

The initiation of blood vessel growth involves focal reduction of intercellular interactions and interactions between the cells of the blood vessel and the surrounding ECM (Lauren et al., 1998). This is associated with a loss of PCs and possibly of SMCs from the existing vessels (Risau, 1997). The maturation of newly formed vessels involves the accumulation of a basal lamina and tightly associated PCs or SMCs on the abluminal side.

In normal adult quiescent vessels, perivascular cells have previously been shown to constitutively secrete Ang-1, enhancing contact between neighbouring ECs and between ECs/perivascular cells, therefore maintaining endothelial integrity and orientation of ECs on the basal lamina leading to vessel stabilisation and maturation of the vasculature (Mandriota et al., 1998; Gamble et al., 2000; Hori et al., 2004).
The presence of Ang-1 and Tie-2 in the diabetic vessels is also consistent with a proposed role for the Ang-1/Tie-2 system at all stages of diabetic retinopathy. Ang-1 is chemotactic for ECs and induces migration, tube formation, sprouting and survival, but not proliferation of ECs in vitro (Davis et al., 1996; Witzenbichler et al., 1998a; Koblizek et al., 1998; Hayes et al., 1999; Kwak et al., 1999; Papapetropoulos et al., 1999; Kim et al., 2000a,b; Kwak et al., 2000). Under conditions of postnatal angiogenesis, Ang-1 expression may be important for initiation of new capillary sprouting, the movement of ECs toward each other and the recruitment of perivascular cells required for fusion into capillary structures (Koblizek et al., 1998; Fujikawa et al., 1999; Kim et al., 2000a, b; Papapetropoulos et al., 2000; Harfouche et al., 2002; Babaei et al., 2003; Harfouche et al., 2003; DeBusk et al., 2004; Metheny-Barlow et al., 2004; Saito et al., 2004). In addition, Ang-1 also has anti-permeability and anti-inflammatory functions (Thurston et al., 1999; Gamble et al., 2000; Thurston et al., 2000; Wang et al., 2000; Kim et al., 2001; Joussen et al., 2002a; Pizurki et al., 2003; Hori et al., 2004; Li et al., 2004; Wang et al., 2004; Jho et al., 2005; Baffert et al., 2006). The observations that Ang-1 and Tie-2 are expressed in endothelial cells of glioblastomas, in leukaemia cell lines, metastatic melanomas and gastric carcinoma (Kaipainen et al., 1994b; Stratmann et al., 1998; Witzenbichler et al., 1998a; Yoshizaki et al., 2004) further supports their role in angiogenesis. Ang-1 has also been shown to promote wound healing through enhanced angiogenesis in a diabetic mouse model (Cho et al., 2006). Tie-2 is also expressed during active vasculogenesis in mice embryos and during vasculogenesis in the developing human placenta (Sato et al., 1993; Schnurch and Risau, 1993; Kayisli et al., 2006). Also, following on from the findings that Ang-1 is expressed in quiescent vasculature during ovulation, Maisonpierre et al. (1997) demonstrated that Ang-1 is also present, alongside that of Ang-2, in late pre-ovulatory follicles and the corpus luteum where angiogenesis is ongoing. Ang-1 when given intravitreally to newly diabetic rats, normalized retinal VEGF and intercellular adhesion molecule-1 mRNA and protein levels, leading to reductions in leukocyte adhesion, endothelial cell injury, and blood-retina barrier breakdown, early pathological changes observed in diabetic retinopathy showing that Ang-1 directly protects the retinal vasculature in diabetes.

Wong et al. (1997) also demonstrated that Tie-2 expression was upregulated in the endothelium of neovessels in rat tissues undergoing angiogenesis during hormonally stimulated follicular maturation and uterine development and in healing skin wounds. However, downregulation of Tie-2 was demonstrated during the later stages coinciding with regression of vessels. This appeared to be apparent in my study where Tie-2 was high in the
preretinal vessels undergoing active angiogenesis but downregulated in the preretinal vessels undergoing regression in the excised membranes. It was also consistent with my finding that Ang-1 was downregulated in the pre-retinal vessels of the excised membranes which supports the observation that the Ang-1 signal is blocked by Ang-2 during vascular regression in ovarian follicles (Maisonpierre et al., 1997).

Ang-2 appears to initiate angiogenesis by binding to the Tie-2 receptor on ECs which results in weakening of the phosphorylation of, and blocking of, the chemotactic effects of Ang-1 (Maisonpierre et al., 1997; Sato et al., 1998; Wittenbichler et al., 1998a). Because Ang-1 is thought to be important for stabilising the vessel wall, local Ang-2 expression might promote SMC/PC drop-off, which is thought to be a requirement for rendering and maintaining ECs accessible to angiogenic inducers. This is supported by the finding that Ang-2 destabilizes quiescent endothelial cells (Scharpfenecker et al., 2004). This loss of PCs was associated with the upregulation of Ang-2 in the ECs of glioblastomas (Stratmann et al., 1998). This role of Ang-2 is also supported by the demonstration that the addition of Ang-2 to VEGF induced neovascularization by promoting vascular destabilisation and sprouting in the corneal micropocket assay (Asahara et al., 1998). Few perivascular cells were present in the vessels supporting the concept that loosening of contacts between ECs and perivascular cells initiates angiogenesis by recruiting VEGF (and possibly other growth factors). Similar findings were observed in the corpus luteum where in the presence of abundant VEGF, Ang-2 may promote vessel sprouting by blocking a constitutive (stabilising) Ang-1 signal, whereas in the absence of VEGF, Ang-2 inhibition of a constitutive Ang-1 signal can contribute to vessel regression (Maisonpierre et al., 1997). Furthermore, Oh et al., 1999 demonstrated that in BRECs VEGF and hypoxia induced an increase in Ang-2. They suggested that the angiogenic stimuli of hypoxia might deteriorate the integrity of the vasculature by suppressing Ang-1 activation of Tie-2. Ang-2 expression has been demonstrated in SMCs, PCs, and microvascular ECs (Mandriota et al., 1998). BMEC Ang-2 mRNA levels were increased by VEGF, VEGF and bFGF in combination, by hypoxia and were decreased by Ang-1 and Ang-2 itself (Maisonpierre et al., 1997; Mandriota et al., 1998; Yamakawa et al., 2003; Pichiule et al., 2004). This suggests that the angiogenic effect of a number of regulators may be achieved in part through the regulation of an autocrine loop of Ang-2 activity in microvascular ECs. That Ang-2 was stimulated by hypoxia further supports its role in pathological diseases characterised by hypoxia. This points to Ang-2 as a potential important component of the angiogenic switch that characterises the passage of diabetic retinopathy from the avascular to the vascular phase, and provides strong evidence for a
collaboration between VEGF and Ang-2 in the regulation of neovascularization in ischaemic tissues. Ang-2 mRNA is also strongly expressed in highly vascularized tumours (Bunone et al., Stratmann et al., 1999; 1999; Brown et al., 2000; Etoh et al., 2001).

My finding that the level of Ang-2 staining was raised in the non-diabetic retinas and the diabetic retinas with no overt retinopathy as compared to the diabetic retinas with vascular changes was unexpected. Ang-2 would be expected to be downregulated in quiescent retina in order to allow Ang-1 to exert its stabilising effects on the vasculature. However, Maisonpierre et al. (1997) also reported the expression of Ang-2 in the normal quiescent wall which suggests a balance of vessel maintenance by positive and negative regulators and the distinct but overlapping expression pattern of Ang-1 and Ang-2 is consistent with the possibility that Ang-2 may regulate Ang-1 function at particular sites and stages of vascular development. Patel et al., 2005 showed that Ang-2 concentrations were higher in the vitreous of patients with NPDR compared to Ang-1 but was found at low concentrations in patients with PDR. The suggested that the levels were lower in the PDR patients due to the established nature of the vitrectomy. That is in long standing and treated PDR, there is little active vessel replication. In established PDR, new vessels are mature and limited active angiogenesis is taking place. They showed that in patients with NPDR the Ang-1 concentrations were half that of Ang-2. Early neovascularization is initiated at this stage of retinopathy as intravascular changes are taking place where changes in Ang-2 levels relative to Ang-1 occur and this allows Ang-2 to predominate at the Tie-2 receptor. Ang-2 is able to competitively inhibit Ang-1 binding to Tie-2. The predominance of the Ang-2 at the Tie-2 receptor would promote increased vascular permeability leading to breakdown of the blood-retinal barrier and neovascularization.

I also demonstrated low levels of Ang-2 in the intra-retinal vessels of the diabetic retinas with vascular changes and in PDR retinas. This finding also contradicts the hypothesised role of Ang-2 as this factor may be expected to be upregulated during these stages of angiogenesis in order to antagonise the vessel stabilising effects of Ang-1. Also Ang-2 is known to promote inflammation and vascular leakage, both features of diabetic retinopathy (Roviezzo et al., 2005). However, it has been shown that Ang-2 may lead to angiogenesis or vessel regression and apoptosis depending on the presence of VEGF (Hanahan, 1997; Lobov et al., 2002). I also encountered some problems with staining using the Ang-1 and Ang-2 antibodies. Stain deposits were apparent on some of the retinal sections which were not removed by centrifuging the antibodies. These observations were less obvious with the Tie-2 antibody. Therefore it must be taken into account that in this study
some non-specific staining may possibly have been observed. Any future studies on immunohistochemistry using the angiopoietin and Tie-2 antibodies should involve some prior treatment of the antibodies (e.g. immunoprecipitation) in order to remove these deposits. Other types of studies such as in situ hybridisation may also produce more reliable results.

In this study Ang-2 was present in the pre-retinal vessels of diabetic retinas undergoing active proliferation which is consistent with its role in angiogenesis. Lower levels in the preretinal vessels of the excised membranes may reflect the fact that they are no longer undergoing active proliferation. This is consistent with the finding that Ang-2 is upregulated during angiogenesis and the progression of hepatocellular carcinoma (Zhang et al., 2006). Also Ang-2 has been shown to stimulate endothelial progenitor cell (EPC) migration to areas of neovascularization (Gill and Brindle, 2005). EPCs have been shown to localize at sites of active angiogenesis and vessel remodelling such as healing wounds, tumours, and ischaemic retina, where they contribute to neovascularization (Asahara et al., 1997; Lyden et al., 2001; Grant et al., 2002).

Ang-2 mRNA was shown to be upregulated in mice models of ischaemia-induced retinal neovascularization, in the INL, GCL, and in the neovascular vessels (Oh et al., 1999; Hackett et al., 2000). When retinal neovascularization started to regress, strong staining was still observed in the GCL and the INL of the hypoxic retinas. Also Ang-2 deficient mice were shown to lack ischaemia-induced retinal neovascularization (Hackett et al., 2002). These data suggest that both hypoxia- and VEGF-induced neovascularization might be facilitated by selective induction of Ang-2 which deteriorates the integrity of the pre-existing vasculature. In my study I also found Ang-2 protein in the GCL and the INL which suggests that the upregulation of Ang-2 precedes the development of neovascularization and parallels the temporal and spatial changes of neovascularization development, which suggests that Ang-2 plays a critical role in retinal neovascularization. Tie-2 was also located in glial cells and ganglion cells. Therefore the angiopoietins appear to have both paracrine and autocrine actions on ECs. Takagi et al., 2002 demonstrated upregulation of Ang-2 and Tie-2 in highly vascularized regions of human epiretinal membranes. Ang-2 were shown to promote tube-forming activity and enhanced the effects of VEGF in cultured BRECs suggesting that in microvascular ECs, Ang-2 can probably induce at least some level of Tie-2 signalling, which contributes to endothelial angiogenic functions.
TNF-α

Immunostaining for TNF-α was performed using a dilution series of a primary antibody from Abcam, against a set number of sections, as recommended by the manufacturer. Initially I found that staining was inconsistent and weak, staining very few sections. I tried changing the dilutions of the antibody, followed by chymotrypsin predigestion but again minimal staining was observed. I repeated the same procedure with a different antibody from Autogen Bioclear but again had the same problems. I then purchased a different antibody from Abcam. This time I tried a dilution series followed by proteolytic predigestion with chymotrypsin but again successful staining wasn’t achieved. Following consultation with the manufacturer I tried 2 different methods of proteolytic predigestion. The first method involved placing the sections in a solution of sodium citrate in a pressure cooker for either 1, 2, or 3 minutes. The second method involved placing the sections in a pressure cooker for 1, 2, or 3 minutes followed by chymotrypsin pre-treatment. I found that placing the sections in the pressure cooker for 3 minutes produced satisfactory results. However following this with chymotrypsin solution damaged the retinas. Therefore for the purposes of this study sections were placed in a pressure cooker for 3 minutes followed by incubation with the primary antibody.

Initially only weak staining was observed for all antibodies. Successful staining was eventually achieved using a highly concentrated dilution of each primary antibody and by exposing the angiogenic binding sites with proteolytic digestion using 0.1% chymotrypsin. In this study low levels of TNF-α were localised to the endothelial and perivascular regions of the non-diabetic retinas and higher levels were demonstrated in diabetic retinas indicating that it may play a role in the pathogenesis of both diabetes and diabetic retinopathy. This is in agreement with Tezel et al., 2001 who demonstrated that TNF-α is constitutively and weakly expressed in normal human retinas.

The highest level of TNF-α immunostaining was observed in the vessels of the diabetic eyes without obvious microvascular changes and was then reduced in retinas with PDR. This supports the finding that high doses of TNF-α have been shown to inhibit angiogenesis whereas low doses were shown to induce angiogenesis (Fajardo et al., 1992). TNF-α is increased during periods of hypoxia (Lahat et al., 2003; Ben-Yosuf et al., 2005) and was more specifically shown to be increased during hypoxia in mouse models of oxygen-induced retinopathy in mice, perhaps before microvascular changes become obvious (Yossuck et al., 2001; Majka et al., 2002). Kerkar et al., 2006 also showed that TNF-α was
able to relax pericyte contractility, which may lead to pericyte dropout, an event which occurs early in diabetes before retinal microvascular changes become obvious.

TNF-α has been shown to play a role in diabetic retinopathy, because it alters the cytoskeleton of ECs, resulting in leaky barrier function and EC activation and an inflammatory response. Studies using diabetic animals have shown that increased leukocyte adhesion to retinal capillaries is an early event in diabetic retinopathy associated with areas of capillary nonperfusion and capillary obstruction and the development of EC damage (Camussi et al., 1991; Kim et al., 1992; Claudia et al., 1994; Deli et al., 1995; Bamforth et al., 1996; de-Vries et al., 1996; Luna et al., 1997; Lutty et al., 1997; Mark et al., 1999; Miyamoto et al., 1999; Joussen et al., 2001; Mark et al., 2001; Mayhan et al., 2002; Trickler et al., 2005; Kerkar et al., 2006; Koss et al., 2006). Joussen et al., 2001 showed that TNF-α in diabetic plasma increases adherence of human leukocytes to retinal ECs, which was increased with the severity of diabetic retinopathy. Menon et al., 2006 showed that TNF-α was able to disrupt VE-cadherin complexes at vascular EC junctions leading to gapping between ECs, causing increased vascular leakage in tumours.

These changes in retinal ECs are central in the progression of diabetic retinopathy. This may explain why it was slightly raised in the diabetic retinas with microvascular abnormalities in my study. The production of TNF-α has been shown to be significantly increased during long-term hyperglycaemia in spontaneously diabetic rats and mice, as well as in streptozotocin-induced diabetic rats. Ages, produced as a result of hyperglycaemia have been shown to promote mRNA expression and secretion of TNF-α in HUVEC (Rashid et al., 2004).

In this study the level of TNF-α expression in the retinal vessels of the PDR retinas was below that observed in the diabetic retinas without microvascular abnormalities but was raised above that seen in the non-diabetic retinas. The level of TNF-α expression was also raised in the neovessels of the PDR retinas and the fibrovascular membranes. This suggests that TNF-α production plays some role in PDR. This is consistent with the findings of Spranger et al., 1995 who demonstrated that there was an increase in TNF-α production in neovascular eye disease and PDR. Levels of TNF-α were shown to be elevated in animal models of ischaemia-induced retinal neovascularization compared with the retinas of non diabetic animals (Yossuck et al., 2001; Joussen et al., 2002c; Majka et al., 2002). TNF-α has previously been detected in the neovessels and extracellular matrix of PDR membranes (Limb et al., 1994; Limb et al. 1996; Armstrong et al., 1998a). Armstrong et al., 1998b showed that oxidative damage associated with tissue hypoxia stimulated retinal
neovascularization in rabbit retina through expression of TNF-α along with VEGF and PDGF. Inhibition of TNF-α or TNF-α knockout was shown to reduce pathological neovascularization in mouse models of oxygen-induced retinopathy by increasing physiological angiogenesis (Gardiner et al., 2005; Kociok et al., 2006). TNF-α has also been found in the vitreous of eyes with PDR (Limb et al., 1991; Franks et al., 1992; Limb et al., 2001) and an association between the serum level of TNF-α and the development of PDR has also been demonstrated (Limb et al., 1996).

TNF-α is a potent inducer of angiogenesis in vivo (Frater-Shroder et al., 1987; Leibovich et al., 1987; Montrucchio et al., 1994). However, in vitro, TNF-α seems to inhibit in vitro angiogenic activities such as endothelial proliferation and tube formation (Frater-Shroder et al., 1987; Sato et al., 1987), suggesting that TNF-α may induce angiogenesis indirectly by activating other regulators of angiogenesis, e.g. inflammatory cell secretion of VEGF, IL-8 etc. TNF-α can induce Tie-2 expression (Willam et al., 2000). Ang-1 is up-regulated by TNF-α (Scott et al 2002; Scott et al., 2005). TNF-α upregulated Ang-2 in HUVECs (Kim et al., 2000d). Therefore TNF-α-induced inflammation angiogenesis might be facilitated by the induction of Ang-2. TNF-α was found in human choroidal neovascular membranes and it colocalized with VEGF, Ang-1, and Ang-2 in cultured choroidal ECs (Hangai et al., 2006). It increased Ang-2 mRNA and protein levels prior to those of Ang-1 and VEGF. These results raise the possibility that during neovascularization, TNF-α may modulate endothelial plasticity and survival by sequential inactivation of Tie-2 followed by activation of Tie-2 and VEGF receptors. Chen et al., 2004 also showed that TNF-α induced a weak angiogenic response in a mouse cornea assay and systemic overexpression of Ang-1 or Ang-2 dramatically increased corneal angiogenesis induced by TNF-α.

In this study the level of TNF-α was raised in the lasered retinas compared to the PDR retinas and was raised significantly compared to the non-diabetic retinas. This is in contrast to other studies in diabetic eyes with intensive coagulation, TNF-α could not be detected (Kutty et al., 1995; Platts et al., 1995).

In this study production of TNF-α was not specifically confined to the retinal vessels. Staining was also observed within the inner retinal layer and the GCL. Staining was also observed within the outer retina in the lasered retinas. This is consistent with the findings of other workers (Fontane et al., 2002; Majka et al., 2002) who showed that in mouse models of retinal ischaemia, TNF-α was found mainly in the inner retina with a stronger expression in the GCL compared with cells of the INL particularly in neurons such as amacrine cells. TNF-α was also detected within structures of the ONL resembling Müller cell processes.
Significant increase of TNF-α expression was observed after reperfusion, in particular the GCL, and to a lesser extent, in INL cells (Fontane et al., 2002) which is consistent with the observation that TNF-α was raised in the lasered retinas in my study. Other workers have shown that retinal Müller cells express TNF-α under hypoxic conditions (Fuchs et al., 2005). TNF-α has been shown to increase the expression of MT1-MMP, MMP-3 and MMP-9 in cultured retinal Müller cells (Migita et al., 1996; Zhang et al., 1998; Majika et al., 2002). MMP-9 has been implicated in cell migration and matrix degradation. The investigators suggested that TNF-α plays a role in the regulation of extracellular proteinase expression during retinal neovascularization. TNF-α binds to ECM proteins, including collagen, fibronectin, and laminin which constitute the main matrix components of epiretinal membranes (Scheiffarth et al., 1988; Alon et al., 1994; Hershkoviz et al., 1995; Franitza et al., 2000). Fontaine et al., 2002 suggested that TNF-α could have neuroprotective or neurotoxic effects in ischaemic retina dependent upon whether it bound to TNF-R1 or TNF-R2.

TNF-α is secreted by glial cells in the CNS and has been shown to be released in activated cultured rat retinal microglia (Morigiwa et al., 2000). Yoshida et al., 2004 showed that TNF-α is produce by activated macrophages/microglia during post-ischaemic inflammation in a mouse model of ischemic retinal neovascularization. Tezel et al., 2001 showed that retinal glial cells weakly stained for TNF-α in normal human retinas. In vitro studies using cocultures of retinal ganglion cells and glial cells showed that TNF-α is upregulated in retinal glial cells after exposure to simulated hypoxia (Tezel et al., 2000).
CHAPTER 5 EXPRESSION OF THE ANTI-ANGIOGENIC GROWTH FACTOR PEDF IN NORMAL AND DIABETIC HUMAN RETINAS.

5.1 INTRODUCTION

To investigate spatial and temporal changes of PEDF expression in the progression of diabetic retinopathy, 25 human retinas were stained with anti-PEDF antibody. 5 non-diabetic retinas and 5 retinas from each of the diabetic groups, summarized in chapter 3 were stained. The intensity of staining was recorded for each retina and an average score calculated for each category of tissue.

5.2 Control Staining

To confirm specificity negative controls were incubated without primary antibody or with primary antibody after pre-absorption with recombinant PEDF. Staining was negative on all the control sections (see figure 5.1).

5.3 PEDF Immunostaining of Retinal Sections

When examined by light microscopy PEDF staining was observed in the non-diabetic retinas (mean 2.2), in retinas without ocular abnormalities (1.6), and in retinas with non-proliferative diabetic retinopathy (1.2) [see figure 5.1]. Intraretinal staining was nearly abolished in patients with PDR (mean 0.4) compared with non-diabetic retinas, retinas with diabetes but without ocular disease and patients with NPDR. Retinas with previous scatter photocoagulation resulting in quiescent PDR had weak intraretinal immunochemical staining that was, on average, slightly more intense than that of the patients with active PDR (mean 1.0).
Figure 5.1 Transverse Sections Showing the Immunolocalisation of PEDF
PEDF immunostaining was localised to non-diabetic retina (A), unlasered diabetic retina with no obvious intraretinal vascular changes (B), unlasered retina with intraretinal vascular changes (C), diabetic retina with PDR (D), and lasered diabetic retina (E). Immunoreactivity was abolished in control retinas processed with normal pig serum (F).
5.4 Discussion

The data presented in this study demonstrate that PEDF was strongly expressed in non-diabetic retinas but that there was almost no staining in retinas with active proliferation. These observations support the view that induction of angiogenesis in diabetic human retinas requires not only elevation of growth factors such as VEGF (Aiello et al., 1994) but also a decrease in angiogenesis inhibitors such as PEDF (Dawson et al., 1999; King et al., 2000).

Several other workers have shown that in normal foetal and adult human and animal retinas, PEDF is expressed in the RPE, photoreceptors, interphotoreceptor matrix (IPM), retinal ganglion cells, cells of the inner retinal layer, nerve fibre layer, inner and outer plexiform layers. (Tombran-Tink et al., 1995; Wu et al., 1995; Ortego, 1996; Wu et al., 1996; Alberdi et al., 1998; Karakousis, 2001; Behling and Bennett, 2002; Ogata et al., 2002bc Eichler et al., 2004; Hattenbach et al., 2005; Becerra, 2006). It has been suggested that secretion of PEDF in the retina, both during vascular development and in adults, accumulates in avascular spaces of the eye such as the aqueous and vitreous humor, and the interphotoreceptor matrix where it is responsible for excluding vessels from invading the retina, vitreous, and cornea (Dawson et al., 1999; Karakousis, 2001; Behling and Bennett, 2002). PEDF has been shown to inhibit VEGF-induced proliferation and migration of microvessel ECs (Duh et al., 2002) and has been shown to inhibit VEGF-induced retinal endothelial cell growth and migration in several animal models of ischaemia induced retinal neovascularization (Mori 2001; Stellmach et al., 2001; Auricchio et al., 2002; Duh et al., 2002; Mori et al., 2002c; Raisler et al., 2002). The mechanism by which PEDF exerts its antiangiogenic action is not fully understood. However, several reports showed that it causes apoptosis of stimulated or proliferating ECs (Stellmach et al., 2001; Guo, 2002; Volpert et al., 2002; Chen et al., 2006) and this may be the mechanism of inhibition of angiogenesis. Cai et al., 2006 demonstrated that PEDF was able to inhibit VEGF-induced angiogenesis via γ-secretase cleavage of VEGFR-1. They also showed that PEDF was also able to inhibit VEGF-induced phosphorylation of VEGFR-1. Phosphorylation of VEGFR-1 was also shown to regulate VEGFR-2 signalling.

The presence of PEDF in the neuronal layers of the retina is also consistent with a role for PEDF as a neuronal survival and neuronal differentiating factor in the retina (Becerra, 1997; Cayouette et al., 1999; Houenou et al., 1999; Jablonski et al., 2000; Cao et al., 2001; Crawford et al., 2001; Ogata et al., 2001c; Becerra et al., 2006; Li et al., 2006). PEDF has been shown to promote photoreceptor outer segment formation and maturation,
and maintains steady state levels of opsin and glutamine synthetase expression in photoreceptor and Müller glial cells (Jablonski et al., 2000; Jablonski et al., 2001).

In this study there was a small reduction in staining intensity in diabetic retinas without microvascular abnormalities and in diabetic patients with NPDR compared with non-diabetic retinas. The finding that PEDF is reduced in patients with NPDR is consistent with the finding that under hypoxic conditions in culture, secretion of PEDF is decreased (Dawson, 1999). It has been shown that PEDF acts as an anti-inflammatory and anti-permeability in the eye (Mori et al., 2001; Stellmach et al., 2001; Liu et al., 2004; Zhang et al., 2005). Yamagishi et al., 2006 showed that PEDF could inhibit AGE-induced retinal vascular hyperpermeability. Down-regulation of PEDF expression has been shown to increase the secretion of VEGF and TNF-α in retinal Müller cells suggesting that a decrease in ocular PEDF levels may play contribute to inflammation and vascular leakage in the eye (Zhang et al., 2005). PEDF has also been shown to protect cultured retinal pericytes from advanced glycation endproduct-induced injury. As pericyte loss from retinal blood vessels is an early feature NPDR a reduction in PEDF, as observed in this study, could lead to pericyte dropout (Yamagishi et al., 1995; Yamagishi et al., 2002).

The finding that there was almost no staining for PEDF in the retinas undergoing active proliferation is consistent with the view that PEDF loss creates a permissive environment for angiogenesis that my contribute to the progression of ocular neovascular disease. Ischaemia- induced retinal neovascularization has been shown to cause a significant reduction in retinal PEDF and a substantial increase in VEGF mRNA and protein in rat models (Gao et al., 2001; Crossen, 2002). A decrease in levels of PEDF has also been described in the vitreous of patients with PDR (Holekamp et al., 2002; Ogata et al., 2001a; 2002a). Vitreous levels of PEDF were shown to be low in eyes with proliferative vitreoretinopathy whereas VEGF levels were raised supporting the view that lower levels of PEDF and higher levels of VEGF may be related to ocular proliferation. However, recently Apte et al., 2004 showed that PEDF stimulated choroidal neovasculariation at high concentrations but at low concentrations it decreased neovascularization. Therefore the concentration of PEDF appears to be important, whether this applies to diabetic retinopathy remains to be determined.

It therefore appears that there is a direct correlation between PEDF and the extent of neovascularization and that there is an equilibrium shift between PEDF and VEGF in the uncontrolled growth of blood vessels in the eye (Gao et al., 2001; Ohno-Matsui et al., 2001; Gao and Ma, 2002; Ogata et al., 2001a; 2002a, b, c). Eichler et al., 2004 showed that
exposure of Müller cells to VEGF suppressed PEDF release in a dose-dependent manner. These findings suggest that in ischaemic retina Müller cells generate a permissive condition for angiogenesis by secreting more VEGF and less PEDF.

More direct evidence for PEDF as an anti-angiogenic factor in PDR comes from studies in which delivery of PEDF protein through virus-mediated gene transfer and direct protein introduction was used successfully to inhibit angiogenesis in a number of animal models of ischaemia-induced retinopathy and choroidal neovascularization (Mori, 2001; Rasmussen, 2001; Stellmach, 2001; Auricchio, 2002; Duh, 2002; Mori et al., 2002ab; Raisler, 2002).

Retinas with quiescent retinal neovascularization who had had retinal photocoagulation had higher levels of PEDF compared with retinas with active PDR. Photocoagulation induces regression of retinal neovascularization and has been shown to be associated with a reduction in the incidence of severe visual loss and retinal neovascularization (Early Treatment Diabetic Retinopathy Study Research Group, 1991). The findings suggest therefore that the positive effects of retinal photocoagulation are mediated at least in part by the re-establishment of near-normal PEDF levels. Hattenbach et al., 2005 observed a considerable upregulation of PEDF mRNA and protein and an increased secretion into the culture medium of RPE cells. PEDF has also been shown to be secreted by RPE cells into the interphotoreceptor matrix of the retina (Siegel et al., 1994; Wu et al., 1995; Wu et al., 1996). Ogata et al., 2001b demonstrated an increased expression of various angiogenic and antiangiogenic cytokines including PEDF mRNA in photocoagulated human RPE cells. The findings from these studies and my study support the hypothesis that antiangiogenic factors play a critical role in the regression of intraocular neovascularization observed after photocoagulation (Diabetic Retinopathy Study Research Group, 1978; Doft et al., 1984).
CHAPTER 6 EXPRESSION OF CAVEOLIN-1, -2, AND -3 IN THE NORMAL AND DIABETIC RETINA

6.1 INTRODUCTION

Sections were stained to localise and assess the extent of caveolin-1 presence in the retina and preretinal membranes. Sections were also stained to localise and assess the extent of caveolin-2 and -3 in normal retinas. Immunostained sections of retina were examined by light microscopy to determine if there was a temporal and spatial relationship between staining intensity and the various pathological changes associated with diabetic retinopathy.

6.2 CONTROL STAINING

To confirm specificity of the immunostaining control sections were processed with omission of the primary antibodies. Staining was negative on all the control sections (see figure 6.1).

6.3 Immunolocalisation of Caveolin-1, -2, and -3

Sections were stained to localise and assess the extent of Caveolin-1, -2, and -3 presence in the retina and preretinal membranes. Immunostained sections of retina were examined by light microscopy to determine if there was a temporal and spatial relationship between staining intensity and the various pathological changes associated with diabetic retinopathy.

6.3.1 Caveolin-1 immunostaining of retinal sections and fibrovascular membranes

When examined by light microscopy staining for caveolin-1 was observed in both non-diabetic and diabetic extravascular tissue. However, caveolin-1 immunoreactivity was generally absent or weak around the intraretinal vessels. Where staining was observed within the vessels, in some instances staining was associated with both endothelial cells and the perivascular region of the vessels. The staining pattern depended upon the category of tissue. Variability of staining within retinal layers and intra-retinal vessels across all categories was observed, with some cells staining negative and some staining positive. Variability of staining was observed for each retina within each category, which is represented by the standard deviations in table 6.1 to 6.6 but this did not show a correlation with either donor age, the type of glycaemic control in the case of diabetic groups or time post-mortem.

In the non-diabetic retinas staining intensity for caveolin-1 was generally minimal or weak within the photoreceptors and the retinal vessels. Only 4/12 retinas demonstrated
staining within the vessels. Weak to moderate staining was observed within the cell bodies of the outer retina (12/12), and the cell bodies of the inner retina (12/12). Moderate staining was observed within the GCL (12/12) [figure 6.1].

In the diabetic retinas with no overt retinopathy minimal to weak staining was again observed within the photoreceptors and the retinal vessels. In comparison to the non-diabetic retinas the number of retinas showing staining for caveolin-1 within the retinas was raised (9/11). As in the non-diabetic retinas, weak to moderate immunoreactivity was associated with the cell bodies of the outer retina (10/11), and the cell bodies of the inner retina (11/11). Staining in the GCL was slightly raised to moderate to intense levels as compared to the non-diabetic retinas (11/11) [figure 6.2].

In the diabetic retinas showing vascular changes but no evidence of PDR staining was raised in the photoreceptors (8/9) and the cell bodies of the inner retina (9/9), as compared with the non-diabetic and diabetic retinas with no overt retinopathy. Staining within the cell bodies of the outer retina was slightly reduced, as compared with the non-diabetic and diabetic retinas with no overt retinopathy (9/9). Staining was again moderate to intense within the GCL (9/9). Staining was still weak within the retinal vessels (5/9) [figure 6.3].

In the diabetic retinas with active neovascular PDR membranes on their surfaces intensity of staining for caveolin-1 was raised in all the retinal layers as compared to all the other previous categories of tissue. Staining was moderate to intense within the photoreceptors (5/5), the cell bodies of the outer retina (5/5), the cell bodies of the inner retina (5/5) and the GCL (5/5). Staining was reduced slightly within the retinal vessels as compared to the other categories of tissue (3/5). Within the pre-retinal membranes staining was weak to moderate within the pre-retinal vessels (5/5) and weak within the non-vascular components of the membranes (4/5) [figure 6.4].

In those diabetic retinas which had undergone successful laser therapy staining was reduced within all the retinal layers as compared to the PDR retinas. Staining was minimal to weak within the photoreceptors (5/11) and the retinal vessels (6/11). Staining was weak to moderate within the cell bodies of the outer retina (10/11), the cell bodies of the inner retina (10/11) and the GCL (10/11) [figure 6.5].

Caveolin-1 staining was weak to moderate within the pre-retinal vessels of 15/17 of the excised membranes. Weak to moderate staining was also associated with the non-vascular components of the membranes [figure 6.6].
### TABLE 6.1. MEAN INTENSITY OF CAVEOLIN-1 IMMUNOSTAINING

<table>
<thead>
<tr>
<th>Tissue Category</th>
<th>Retinal Layer</th>
<th>Retinal Vessels</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Photo-Receptors</td>
<td>Outer Retina</td>
<td>Inner Retina</td>
</tr>
<tr>
<td>Non-diabetic (n=14)</td>
<td>0.9</td>
<td>1.6</td>
<td>1.9</td>
</tr>
<tr>
<td>No Overt Retinopathy (n=12)</td>
<td>0.7</td>
<td>1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Intraretinal Changes (n=10)</td>
<td>1.3</td>
<td>1.1</td>
<td>2.2</td>
</tr>
<tr>
<td>PDR (n=9)</td>
<td>2.2</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Laser-No Residual PDR (n=14)</td>
<td>0.9</td>
<td>1.1</td>
<td>1.9</td>
</tr>
<tr>
<td>Excised Membranes (n=17)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

GCL = ganglion cell layer  
ILM = internal limiting membrane

0 = background staining  
1 = weak staining  
2 = moderate staining  
3 = intense staining

Values in parenthesis = +/- standard deviation
6.3.2 Caveolin-2 Immunostaining of retinal sections

Of the 8 non-diabetic retinas tested, staining was absent in the RPE, however staining was observed in the outer segments of the photoreceptors in 7/8 of the retinas. Staining was also observed in the outer region of 2/8 of the retinas, in the inner region of 3/8 of the retinas and in the ganglion cell layer of 4/8 of the retinas. Perivascular staining was observed in 3/8 of the retinas and in one of the retinas one vessel showed endothelial cell staining [figure 6.7].

6.3.3 Caveolin-3 immunostaining of retinal sections

Of the 8 non-diabetic retinas tested only one stained positively for caveolin-3 where immunostaining was localised to the outer and inner regions of the retina and the ganglion cell layer [figure 6.7].
Figure 6.1 Transverse Sections Showing the Immunolocalisation of Caveolin-1 in Non-Diabetic Retinas (A-E) and Negative Control Section (F).
Staining was observed in the retinal vessels and across all the retinal layers.
Figure 6.2 Transverse Sections Showing the Immunolocalisation of Caveolin-1 in Unlased Diabetic Retinas with No obvious Microvascular Abnormalities. Staining was observed in the retinal vessels and across all the retinal layers.
Figure 6.3 Transverse section showing the immunolocalisation of Caveolin-1 in Unlasered Diabetic Retinas with No obvious Microvascular Abnormalities.
Staining was observed in the retinal vessels and across all the retinal layers.
Figure 6.4 Transverse Sections Showing the Immunolocalisation of Caveolin-1 in Diabetic Retinas with PDR.
Staining was observed in the retinal vessels and across all the retinal layers.
Figure 6.5 Transverse Sections Showing the Immunolocalisation of Caveolin-1 in Lasered Diabetic Retinas. Staining was observed in the retinal vessels and across all the retinal layers.
Figure 6.6 Transverse Sections Showing the Immunolocalisation of Caveolin-1 (Arrows) in Fibrovascular Membranes
Figure 6.7 Transverse Sections Showing the Immunolocalisation of Caveolin-2 and Caveolin-3
Caveolin-2 immunostaining was localised to non-diabetic retina (A, B). Caveolin-3 immunostaining was localised to non-diabetic retina (C,D). Immunoreactivity was abolished in control retina processed with normal goat serum (E).
6.4 DISCUSSION

Immunostaining for caveolin-1, caveolin-2 and caveolin-3 was performed using a dilution series of the primary antibodies, against a set number of sections, as recommended by the manufacturer. Initially only weak staining was observed for all antibodies. Proteolytic digestion using 0.1% chymotrypsin or proteinase K also only produced weak staining. Successful staining was eventually achieved using a highly concentrated dilution of each primary antibody and by exposing the angiogenic binding sites with proteolytic digestion using 0.2% triton X-100. I also found that there was a lot of background staining for caveolin-1 using the alkaline phosphatase/fast red system. I therefore substituted that enzyme/substrate complex for horseradish peroxidase/diaminobenzidine which produced more specific staining.

The data presented in this study demonstrate that caveolin is present in human retina. In addition caveolin-1 was shown to be present in diabetic retinas and fibrovascular membranes. These observations support a role for caveolin in the normal functioning of the retina and in diabetic retinopathy.

Caveolin-1 was present at low levels in the retinal vessels of the non-diabetic retinas and at all stages of diabetic retinopathy. Staining was slightly raised in the neovessels of the microvascular membranes of the PDR retinas. Several workers have demonstrated caveolin-1 expression in retinal endothelial cells (Gardiner and Archer, 1986a, b; Feng et al., 1999b; Bridges et al., 2001; Kim et al 2006) indicating that it is involved in the normal physiological functioning of the retina.

Cav-1 and caveolae have been shown to serve as flow-activated mechanosensors or transducers of physiological responses in intact blood cells (Yu et al., 2006). Caveolae are also believed to be responsible for transcytosis, the process by which plasma proteins (e.g. albumin) are transported across capillary endothelium (Palade et al., 1979; Vasile et al., 1983; Ghitescu et al., 1986; Milici et al., 1987; Schnitzer et al., 1995; Predescu et al., 1998).

Cav-1 is the major protein coat of endothelial caveolae and is necessary for caveolae assembly. Several studies have shown a complete loss of caveolae organelles in blood vessels, adipocytes and fibroblasts obtained from cav-1-deficient mice confirming the necessity of this protein in caveolae biogenesis (Drab et al., 2001; Razani et al., 2001; Rizani and Lisanti, 2001; Zhao et al., 2002; Cao et al., 2003). Cav-1 appears to play an important role in cholesterol homeostasis and caveolae has been shown to play a role in cholesterol trafficking as has been observed within lens epithelial cells and fibre cells (Lo et al., 2001; Sexton et al., 2004; Cenedealla et al., 2006).
Caveolae are able to form mobile signalling platforms which concentrate and organize signal transduction cascades that regulate tissue structure, vascular tone, and basal permeability (Anderson, 1993; Lisanti et al., 1994; Parton, 1996). In caveolae, cav-1 can interact with itself to form homo-oligomers as well as with several other signalling molecules.

Caveolae have been implicated in signalling through the p42/44 MAP kinase pathway which is involved in the proliferation, survival, and migration of ECs. Other components of the p42/MAP kinase cascade are localized within caveolae membranes. These include receptor tyrosine kinases (VEGFR-2, Tie-2, EGFR, PDGFR, InsR) (Smart et al., 1995; Liu et al., 1996; Mineo et al., 1996; Liu et al., 1997; Liu et al., 1999; Cho et al., 2004b; Sonveaux et al, 2004), H-Ras (Mineo et al., 1996; Song et al., 1996; Yoon et al., 2003), Raf kinase (Mineo et al., 1996), ERK (Lisanti et al., 1994; Liu et al., 1996), Shc (Liu et al., 1996), Grb-2 (Liu et al., 1996) and Nek (Liu et al., 1996) as well as PI-3 kinase, PLCγ and protein kinase C and phosphatidylinositol (Lisanti et al., 1994; Liu et al., 1995; Pike et al., 1996; Liu et al., 1997; Gingras et al., 1998; Okamoto et al., 1998; Ahn et al., 1999; Murthy and Makhlouf, 2000; Predescu et al., 2001; Kawamura et al., 2003; Labrecque et al., 2003;). Both in vitro and cell culture experiments indicate that cav-1 can directly interact with and maintain some of these signalling molecules in an inactive conformation (Anderson, 1998; Okamoto et al., 1998) acting as a scaffolding protein, able to negatively regulate the activity of other molecules by binding to and releasing them in a timely fashion holding them in a quiescent or inhibited state. Down regulation of cav-1 expression and caveolae organelles may be a prerequisite for EC proliferation and subsequent angiogenesis. This is supported by the finding that exposure of Ecs to VEGF, has been shown to result in a reduced EC expression of cav-1 and caveolae which was mediated by a negative feedback mechanism through the VEGFR-2 and subsequent downstream p42/44 MAP kinase pathway (Liu et al., 1999; Labrecque et al., 2003). Cav-1 overexpression has been shown to reduce VEGF-mediated angiogenesis with defects in vasodilatation, permeability, and angiogenesis (Brouet et al., 2004; Bauer et al., 2005). Sonveaux et al., 2004 showed that cav-/- Ecs formed tubes on Matrigel on VEGF stimulation which was dramatically repressed when compared with cav+/- Ecs. With the exception of insulin receptor which appears to be stimulated by cav-1 (Yamamoto et al., 1998), studies have demonstrated that cav-1 bound to and inhibited growth factor receptor kinase activity (Couet et al., 1997; Bilderback et al., 1999; Yamamoto et al.,
1999) as well as other important secondary messengers and substrates (Shaul and Anderson, 1998).

Liu et al., 2002 showed that cav-1 is down-regulated by endothelial growth factors that stimulate the initial proliferative phase but upregulated during the differentiation phase into tubular networks. Under conditions of cell confluence, they showed that cav-1 expression stimulates EC differentiation and tubule formation. They hypothesize that caveolin acts as both a pro- and anti-angiogenic factor by serving as a “differentiation sensor” that monitors and responds to changes in the relative balance of positive and negative factors to “tell” a target cell whether to remain quiescent or to become proliferative. This may explain why I observed the same levels of cav-1 staining across all tissue categories. In the PDR membranes the neovessels may have already undergone proliferation and so cav-1 may now be acting as an anti-angiogenic factor.

I also observed cav-1 staining in the photoreceptors, the ganglion cell layer and the inner and outer regions of both non-diabetic and diabetic retinas. Interestingly levels were raised in the diabetic retinas as compared to the non-diabetic retinas. Kim et al., 2006 also observed cav-1 immunostaining in the photoreceptors, ganglion cell layer, the inner plexiform layer and outer plexiform layer of normal rat retinas which is in agreement with my findings. This may indicate that cav-1 plays a role in signal transduction in glial and neuronal cells. As far as I am aware, no other studies have looked at the expression of cav-1 in the diabetic retina and so its role in diabetic retinopathy is still to be determined. Photoreceptor detergent resistant membranes (DRMs) are enriched in cav-1 (Elliott et al., 2003) and cav-1 has been shown to be localized to photoreceptor synaptic ribbons. DRMs from rod outer segments contain several proteins involved in phototransduction (Seno et al., 2001). It was suggested that cav-1 may therefore be involved with regulating transmitter release and visual transduction and the formation of synaptic ribbons (Kachi et al., 2001).

In my study I observed cav-2 staining in the photoreceptors, the neuronal layers of the retinas and the ganglion cell layer of non-diabetic retinas, some staining was also observed around the vessels. As well as cav-1 expression endothelial cells also express cav-2 but not cav-3 (Garcia-Cardena et al., 1997). Cav-2 has to heteroligomerize with cav-1 to form the caveolar coat but cannot form the caveolar coat by itself (Monier et al., 1995; Sargiacomo et al., 1995; Scherer et al., 1997; Scheiffele et al., 1998; Mora et al., 1999; Parolini et al., 1999). Cav-2 has been shown to be expressed in adult rat retina where it showed similar but weaker staining than cav-1 (Kim et al., 2006) but staining was more intensely detected in vessels as compared to cav-1. As in my study cav-2 was present in the inner plexiform layer,
inner nuclear layer, outer plexiform layer in the processes of glial cells and Müller cells. Also in the retinal neuronal cells including the ganglion cells, amacrine cells, bipolar cells, horizontal cells, and photoreceptor cells. Based on the functional role of caveolins, these molecules may be assumed to play an important role in the cholesterol homeostasis of the retina. Cav-2 may also be involved in synaptic signal transduction in the retina and cav-2 has also been shown to be expressed in brain astrocytes (Cameron et al., 1997; Ikezu et al., 1998). Cav-2 has also been shown to be present in the RPE collected from rat eyes and in human RPE cell lines (Mora et al., 2006) which was in contrast to my study where immunostaining was not observed. Cav-2 has a non-polar distribution in RPE cells, consistent with the observation that cav-2 expression may be necessary in certain cells for the efficient assembly of plasmalemmal caveolae (Lahtinen et al., 2003; Sowa et al., 2003). The determination of the precise role of cav-2 in the retina will require further study.

Cav-3 is primarily expressed on skeletal muscle and smooth muscle cells (Song et al., 1996; Tang et al., 1996; Kogo et al., 2006). In my study I showed that in one retina, cav-3 was detected in the outer and inner regions of the retina and the ganglion cell layer indicating that it is expressed by cells other than muscle cells. Cav-3 was also shown to be expressed in brain astroglial cells (Ikezu et al., 1998) and has been shown to be upregulated in reactive astrocytes surrounding senile plaques in the brains of Alzheimer's patients (Nishiyama et al., 1999). Cav-3 shows high homology with cav-1 (85% similarity) (Tang et al., 1996) and either is sufficient for the formation of caveolae invaginations (Li et al., 1998; Hagiwara et al., 2000; Galbiati et al., 2001; Park et al., 2002). Cav-3 can interact with adenyl cyclase, eNOS and insulin receptor signalling (Yamamoto et al., 1998).

There is therefore some indication that cav-2 and cav-3 are involved both in the normal physiological functioning of the retina, as shown by my study, and in various disease processes but further studies need to be carried out to determine their precise roles.
7. GENERAL DISCUSSION

7.1 MICROVASCULAR COMPLICATIONS AND DIABETIC RETINOPATHY

Retinopathy is a frequent microvascular complication of diabetes mellitus (DM) that remains a major therapeutic challenge (Lobo et al., 2004). To prevent and improve the treatment of DR, it is fundamental that we know the evolution of the earliest changes that occur in the retina affected by DM and how these changes relate to the progression of retinopathy. In this study retinas were selected for immunostaining based on clinical assessment and by microscopical examination of sections for microvascular abnormalities associated with diabetic retinopathy.

The overall purpose of this project was to try to demonstrate that the spatial and temporal expression of angiogenic growth factors was altered during the progression of diabetic retinopathy. In recent years it has become increasingly obvious that inhibitors of angiogenesis are just as important as pro-angiogenic factors in the pathogenesis of diabetic retinopathy. I therefore also examined the expression of the well known anti-angiogenic factor PEDF during different stages of diabetic retinopathy. Finally, this study was also undertaken to determine if the levels of caveolin changed during the progression of diabetic retinopathy as caveolae have been shown to play an important role in the compartmentalization of second messengers involved in growth factor receptor signal transduction pathways.

7.2 GROWTH FACTOR EXPRESSION DURING DIABETIC RETINOPATHY

The findings from my study clearly show that VEGF, the angiopoietins and TNF-α play different roles in the pathogenesis of diabetic retinopathy. It therefore appears that growth factors may act alone or in concert to bring about the microvascular abnormalities observed in diabetic retinopathy.

The specific localization of VEGFR-2 in PDR retina supports the previous suggestion that VEGF binding to the VEGFR-2 receptor sends a classic proliferative signal (Malecaze et al., 1994; Chen et al., 1997; Armstrong et al., 1998a; Suzuma et al., 1998; Ishida et al., 2000; Ozaki et al., 2000; Ishimama et al., 2001; Witmer et al., 2002; Gerber and Ferrara., 2003; Cerdan et al., 2004; Wilkinson-Berka et al., 2006). The demonstration that VEGFR-1 is expressed in both non-diabetic retina and diabetic retina supports the concept that VEGF is involved in vascular maintenance as well as in other endothelial cell functions such as maintenance of vascular permeability and endothelial cell proliferation (Kolch et al., 1995).
This study adds to the limited knowledge about the role of VEGFR-3 in pathological angiogenesis (Pajusola et al., 1992; Fielder et al., 1997; Partanen et al., 1999; Valtola et al., 1999; Skobe et al., 2001ab; Witmer et al., 2001; Clarijs et al., 2002). VEGF-C can induce angiogenesis under certain circumstances which could be due in part to its capacity to bind to an activate VEGFR-2, in addition to VEGFR-3 (Tille et al., 2003). Clearly VEGF-C binding to VEGFR-3 does play a role in diabetic retinopathy.

VEGF increases permeability and is mitogenic for ECs, acting early and at most points of the angiogenic cascade (Lim et al., 2003). In contrast, Ang-1 has vessel-maturing activates and acts at a later stage which involves recruitment of vessel-supporting cells, strengthening of intercellular junctions, and establishment of leakage-resistant vessels (Suri et al., 1996; Davis and Yancopoulos, 1999; Fujikawa et al., 1996b; Hanahan, 1997; Papapetropoulas et al., 1999; Thurston et al., 1999; Gamble et al., 2000). It has been shown that a combination of Ang-1 and VEGF recruits smooth muscle actin-α-positive cells to vascular walls (Asahara et al., 1998). Co-induction of endothelial Ang-1 and VEGF may exert such vessel-maturing effects on the sprouting vessels in an autocrine manner in the microenvironment of active sprouting. Ang-1 exerts a vascular endothelial barrier protective effect by blocking the action of VEGF (Gamble et al., 2000; Jho et al., 2005). I did find low levels of Ang-1 in PDR membranes which supports this role of Ang-1 however, a high level of Ang-1 immunostaining was observed in the PDR retinal vessels. Perhaps it was localized to a subset of retinal vessels that were no longer undergoing active angiogenesis. However in the presence of VEGF, Ang-1 has been shown to significantly potentiate VEGF-induced neovessel sprouting (Zhu et al., 2002).

Ang-2 signals cause vessel structures to become loosened, reducing endothelial cell contacts with the matrix and disassociation of perivascular support cells. This loosening appears to render the endothelial cells more accessible and responsive toward angiogenic inducers such as VEGF (and likely other angiogenic inducers) leading to capillary sprouting and the formation of new vessels. I only showed low levels of Ang-2 expression in the retinal vessels which was an unexpected finding. Again, perhaps it was localized to a subset of vessels that were not undergoing active angiogenesis. The action of Ang-2 has become controversial as in some instances Ang-2 can either promote angiogenesis or induce regression of vessels (Maisonpierre et al., 1997; Holash et al., 1999; Yu and Stamenkoviv, 2001). This appears to be dependent upon the presence of VEGF. When VEGF is present, Ang-2 appears to induce angiogenesis, but in the absence of VEGF it promotes vessel regression (Asahara et al., 1998; Lobov et al., 2002; Oshima et al., 2004). Hangai et al., 2006
speculate that sequential induction of Ang-2 and then Ang-1 and VEGF in ECs may provide precise and stage-appropriate autocrine and/or paracrine angiogenic signals to ECs.

The findings from this study that TNF-α was upregulated in the vessels of PDR membranes and diabetic retinas supports the suggestion that it may also play some role in diabetic retinopathy (Spranger et al., 1995; Limb et al., 1996; Armstrong et al., 1998a; Limb et al., 2001; Yossuck et al., 2001; Majka et al., 2002). Studies of the angiogenic properties of TNF-α yield conflicting results. In vivo it appears to be a potent inducer of angiogenesis (Frater-Shroder et al., 1987; Leibovich et al., 1987; Montrucchio et al., 1994). However, in vitro, TNF-α seems to inhibit angiogenic activities such as endothelial proliferation and tube formation (Frater-Shroder et al., 1987; Sato et al., 1987; Yang et al., 2004), apart from in one study in HUVECs where TNF-α was shown to upregulate Ang-2 expression (Kim et al., 2000d). It has been suggested that TNF-α may induce angiogenesis indirectly by activating other regulators of angiogenesis. TNF-α has been shown to induce Ang-1, Ang-2, Tie-2 VEGF, and VEGF-C expression (Kim et al., 2000d; Willam et al., 2000; Scott et al., 2002; Chen et al., 2004; Scott et al., 2005; Hangai et al., 2006; Zhao et al. 2006; Zhu et al., 2006). Results from our laboratory also showed that TNF-α was also able to upregulate KDR (Zhao et al., 2006). TNF-α was also shown to co-localize with VEGF and the angiopoietins in choroidal neovascular membranes and it increased Ang-2 levels prior to those of Ang-1 and VEGF. This suggests that during neovascularization TNF-α may modulate endothelial plasticity and survival by sequential inactivation of Ang-2 followed by activation of Tie-2 and the VEGF receptors.

VEGF, Ang-2 and TNF-α have been shown to be hypoxia inducible (Oh et al., 1999). As VEGF, the angiopoietins and TNF-α were localized to vascular endothelial cells and non-vascular cells this supports the hypothesis that the retinal response to the hypoxic environment would be to upregulate the receptors and to stimulate the synthesis and secretion of VEGF, Ang-2 and TNF-α in retinal PCs, ECs, glial cells, ganglion cells, and possibly other cell types. The sustained production of VEGF, Ang-1, Ang-2 and TNF-α would eventually lead to an angiogenic response. Sustained production of these growth factors may be maintained by a positive feedback mechanism to the receptors on the retinal cells. The presence of Ang-1 in retinal vessels not involved in retinal angiogenesis may allow a shift in the local balance of Ang-1/Ang-2 back in favour of Ang-1, to effect maturation and stabilization of the newly formed vessels. Therefore there appears to be a collaboration between VEGF, Ang-1, Ang-2, and TNF-α to elicit angiogenesis, all of which appear to exert their effects on retinal cells via both autocrine and paracrine mechanisms.
7.3 THE EXPRESSION OF PEDF IN DIABETIC RETINOPATHY

The findings from this study support the findings of other workers that PEDF is highly expressed in non-diabetic retinas and diabetic retinas with NPDR. (Tombran-Tink et al., 1995; Wu et al., 1995; Ortego, 1996; Wu et al., 1996; Alberdi et al., 1998; Karakousis, 2001; Behling and Bennett, 2002; Ogata et al., 2002bc; Eichler et al., 2004; Hattenbach et al., 2005; Becerra, 2006). This study also showed that PEDF is downregulated in PDR which confirms findings obtained from previous studies (Holekamp et al., 2002; Ogata et al., 2001a; 2002b, c).

It appears therefore that under normal physiological conditions, secretion of PEDF into avascular spaces in the eye such as the aqueous and vitreous, excludes vessels from invading the retina, vitreous and cornea (Dawson et al., 1999; Karakousis, 2001; Behling and Bennett, 2002). It may do this by inhibiting VEGF-induced proliferation and migration of ECS (Mori 2001; Stellmach et al., 2001; Auricchio et al., 2002; Duh et al., 2002; Mori et al., 2002c; Raisler et al., 2002; Cai et al., 2006).

The general consensus is that loss of PEDF creates a permissive environment for angiogenesis that may contribute the progression of ocular neovascular disease. This is supported by the findings that ischaemia-induced retinal neovascularization caused a significant reduction in retinal PEDF and a substantial increase in VEGF mRNA and protein in rat models (Gao et al., 2001; Crossen, 2002). Down-regulation of PEDF expression has also been shown to increase the secretion of both VEGF and TNF-α from retinal Müller cells (Zhang et al., 2005). It therefore appears that there is a direct correlation between PEDF and the extent of neovascularization and that there is an equilibrium shift between PEDF and VEGF in the uncontrolled growth of blood vessels in the eye (Gao et al., 2001; Ohno-Matsui et al., 2001; Gao and Ma, 2002; Ogata et al., 2002a, b, c; Eichler et al., 2004). However, recently Apte et al., 2004 showed that PEDF stimulated choroidal neovasculariation at high concentrations but at low concentrations it decreased neovascularization. Therefore the concentration of PEDF appears to be important, whether this applies to diabetic retinopathy remains to be determined and therefore caution should be taken when considering using PEDF for the treatment of PDR.

7.4 THE EXPRESSION OF CAVEOLINS IN DIABETIC RETINOPATHY

The observation that only cav-1 and cav-2 were observed in the retinal vessels is consistent with the findings from other studies where cav-1 and cav-2 have both been shown to be expressed by retinal endothelial cells but not cav-3 (Garcia-Cardena et al., 1997; Feng
et al., 1999b; Bridges et al., 2001; Kim et al., 2006). The presence of cav-1 and cav-2 in non-diabetic retina supports previous observations that under normal physiological conditions caveolae act as flow-activated sensors in ECs (Yu et al., 2006). Shear stress is recognized by the endothelial cells and regulates vascular tone, vessel wall remodelling, cell adhesion, coagulation, and fibronolysis (Dewey et al., 1981; Davies et al., 1995). Caveolae are also believed to be involved in transcytosis, the process by which proteins (e.g. albumin) are transported across capillary endothelium (Palade et al., 1979; Vasile et al., 1983; 1989; Predescu et al., 1998). In addition cav-1 and cav-2 hetrodimerize to form the protein coat of caveolae and both are necessary for caveolae assembly (Drab et al., 2001; Razani et al., 2002).

As well as their physiological roles in vascular endothelium, caveolae are able to form mobile signalling platforms which concentrate and organize signal transduction cascades. (Anderson, 1993; Lisanti et al., 1994; Parton, 1996). Several receptor tyrosine kinases are located in caveolae including VEGFR-2 and Tie-2 as well as the TNF receptors I and II (Feng et al., 1999a; Liu et al., 1999; Yoon et al., 2003; Cho et al., 2004b; D’Allessio et al., 2005). Cav-1 has been shown to interact with and maintain some of these signalling molecules in an inactive conformation acting as a scaffolding protein, able to negatively regulate the activity of these molecules by binding to and releasing them in a timely fashion holding them in a quiescent or inhibited state. Down regulation of cav-1 may be a prerequisite for EC proliferation and subsequent angiogenesis. Down-regulation of cav-1 was demonstrated in the retinal vessels of the PDR retinas in this study and exposure of ECs to VEGF has been shown to result in a reduced EC expression of cav-1 which was mediated by a negative feedback mechanism through the VEGFR-2 receptor and subsequent downstream p42/44MAP kinase pathway (Liu et al., 1999; Labrecque et al., 2003). This does not support the findings that cav-1 expression was increased in the neovessels of the PDR retinas. These conflicting findings for cav-1 expression may be explained by the fact that cav-1 expression appears to be down-regulated during the proliferative phase of angiogenesis, and then markedly upregulated during the differentiation phase (Liu et al., 2002). Liu et al., 2002 showed that cav-1 is downregulated by endothelial growth factors that stimulate the initial proliferative phase but upregulated during the differentiation stage into tubular networks. They hypothesized that caveolin acts a both a pro- and anti-angiogenic factor by serving as a “differentiation sensor” that monitors and responds to changes in the relative balance of positive and negative factors to “tell” a target cell whether to remain quiescent or to become
proliferative. In my study in the PDR membranes the neovessels may have already undergone proliferation and so cav-1 may now be acting as an anti-angiogenic factor.

The presence of the caveolins in the ganglion cells and glial cells agrees with the findings of other workers and may indicate that the caveolins play some role in signal transduction in these cells. (Kachi et al., 2001).

7.5 FUTURE WORK

Examination of immunostained retinal sections by light microscopy provided only limited information on the exact cellular localization of these growth factors and their receptors in diabetic retinopathy. Further studies at the ultrastructural level using either electron microscopy or confocal microscopy should help. It would also have been helpful to undertake staining of all the diabetic tissue with cav-2 and cav-3 antibodies as this will further clarify their roles in diabetic retinopathy and also with antibodies to TNF receptors I and II. This should be supplemented with in situ hybridization studies which would provide information on the exact cellular localization of cytokine production.

The current methods of treatment for PDR, including laser photocoagulation, vitrectomy and cryotherapy are expensive and cause destruction of viable retinal tissue. Non-invasive therapies are currently being sought to replace these surgical interventions. Over the last decade much research has concentrated on using inhibitors of the VEGF signalling pathway for the treatment of retinal neovascular diseases such as PDR and age related macula degeneration (AMD) and some success has been achieved using aptamers and antibodies which bind VEGF (Afzal et al., 2007). Magugen®, an oligonucleotide aptamer and Lucentis®, a humanized antibody fragment have both been approved by the FDA for use in reversing the neovascularization associated with AMD and have been used successfully to treat macula oedema in clinical trials (Brown et al., 2006; Rosenfield et al., 2006). However these molecules are large and complex, are difficult to administer intravitreally and the regression of neovascularization is rarely permanent, which requires multiple applications of drug which is expensive. Bevacizumab (Avastin®), a humanized full-length antibody against VEGF, is a less expensive alternative which has been used in clinical trials for diabetic retinopathy and diabetic macula oedema where it was shown to exert its effects by blocking increased vascular permeability and angiogenesis (Averbukh et al., 1998; Cunningham et al., 2005). Several reports describe the use of off-label Bevacizumab in various proliferative retinopathies with subsequent good visual and structural outcomes (Abraham-Marin et al., 2006; Rich et al., 2006). Various molecules which are smaller, can be administered less
invasively and are less expensive are currently under investigation. Theses include Vatalanib (VEGFR tyrosine kinase inhibitor) (Steeghs et al., 2007), Sirna-027 (SiRNA which down regulates VEGFR-1), VEGFR-SiRNA (SiRNA which down-regulates VEGF) [Reich et al., 2003] and T2-TrpRS (a proteolytic fragment of tryptophan tRNA synthetase) [Banin et al., 2006].

Precise understanding of the biologic roles of the VEGF, the angiopoietin family members, TNF, PEDF and caveolins and of how they may collaborate with each other and other growth factors may lead to novel and therapeutically significant strategies for promoting or inhibiting neovascularization. However, development of long-term therapeutic interventions based on the blocking of Tie-2, VEGFR-1, and possibly VEGFR-3, signalling should proceed with caution due to the possible disruption of crucial roles of these receptors in vascular maintenance. Further investigation into the specific signalling pathways used for vascular growth and maintenance may also lead to more specific and effective strategies for therapy.
<table>
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<tr>
<th>Abbreviation</th>
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<td>α</td>
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<td>Ab</td>
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<td>ABCComplex</td>
<td>Avidin-Biotinylated Alkaline Phosphatase Complex</td>
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<td>Advanced Glycation End Products</td>
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<td>Ang-1</td>
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<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
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<td>Basement Membrane</td>
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<tr>
<td>BMEC</td>
<td>Bovine Microvascular Endothelial Cell</td>
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<td>BREC</td>
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<tr>
<td>BRPC</td>
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<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
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<td>ELM</td>
<td>External Limiting Membrane</td>
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<td>FGF</td>
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<td>GABA</td>
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<td>IMS</td>
<td>Industrial Methylated Spirit</td>
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<td>kb</td>
<td>Kilobase</td>
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<td>KDR</td>
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<td>mg</td>
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<td>mm</td>
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<td>Abbreviation</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<tr>
<td>min</td>
<td>Minute</td>
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<tr>
<td>NBF</td>
<td>Neutral Buffered Formalin</td>
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<td>NDRI</td>
<td>National Disease Research Interchange</td>
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<tr>
<td>NO</td>
<td>Nitric Oxide</td>
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<td>NPDR</td>
<td>Non-proliferative Diabetic retinopathy</td>
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<td>PLC-γ</td>
<td>Phospholipase C-gamma</td>
</tr>
<tr>
<td>pO2</td>
<td>Oxygen Tension</td>
</tr>
<tr>
<td>PSMC</td>
<td>Porcine Smooth Muscle Cell</td>
</tr>
<tr>
<td>RGC</td>
<td>Retinal Glial Cell</td>
</tr>
<tr>
<td>ROP</td>
<td>Retinopathy of Prematurity</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal Pigmented Epithelium</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>sFlt-1</td>
<td>Soluble Fms-like Tyrosine Kinase-1</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth Muscle Cell</td>
</tr>
<tr>
<td>TEK</td>
<td>Tunica Interna Endothelial Cell Kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Tie-2</td>
<td>Tyrosine Kinase With Ig-like loops and epidermal growth factor homology domains-2</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming Growth Factor-Alpha</td>
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<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-Beta</td>
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<tr>
<td>TNF-α</td>
<td>Tumour Necrosis Factor-Alpha</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase-Type Plasminogen Activator</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>VAS</td>
<td>Vasculotropin</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>VEGFR</td>
<td>Vascular Endothelial Growth Factor Receptor</td>
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<tr>
<td>VPF</td>
<td>Vascular Permeability Factor</td>
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<tr>
<td>v/v</td>
<td>Volume by Volume</td>
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</table>
APPENDIX I: MATERIALS AND SUPPLIERS

ABComplex/Alkaline Phosphatase  DAKO
Aminopropyltriethoxysilane  Sigma
Anti-Angiopoietin-1 Antibody  Santa Cruz
Anti-Angiopoietin-2 Antibody  Santa Cruz
Anti-Caveolin-1 Antibody  BD Transduction Laboratories
Anti-Caveolin-2 Antibody  BD Transduction Laboratories
Anti-Caveolin-3 Antibody  BD Transduction Laboratories
Anti-TNF-α Antibody  Abcam
Anti-VEGFR-1 Antibody  Santa Cruz
Anti-VEGFR-2 Antibody  Santa Cruz
Anti-VEGFR-3  Santa Cruz
Anti-Tie-2 Antibody  Santa Cruz
Anti-VEGF165 Antibody  Santa Cruz
Anti-VEGF-C Antibody  Santa Cruz
Biotinylated Goat Anti-Rabbit Immunoglobulin (IgG)  Sigma
Biotinylated Rabbit Anti-Goat Immunoglobulin (IgG)  Sigma
Chloroform  BDH
α-Chymotrypsin  Sigma
Cover Slips  Raymond Lamb
Disodium Hydrogen Orthophosphate  BDH
Eosin  BDH
Fast Red/TR Naphol AS-MX Tablets  Sigma
Filters (0.2μm)  BDH
Formaldehyde  BDH
Glass Microscope Slides  Raymond Lamb
Haematoxylin(Harris and Mayers)  BDH
Hydrochloric Acid  Sigma
Hydromount  National Diagnostics
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
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<tr>
<td>Industrial Methylated Spirit</td>
<td>Genta Medical</td>
</tr>
<tr>
<td>Loctite Adhesive</td>
<td>Till and Whitehead</td>
</tr>
<tr>
<td>Milk Protein</td>
<td>Premier Beverages</td>
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<tr>
<td>Normal Goat Serum</td>
<td>Sigma</td>
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<tr>
<td>Normal Pig Serum</td>
<td>Sigma</td>
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<tr>
<td>Normal Rabbit Serum</td>
<td>Sigma</td>
</tr>
<tr>
<td>Paraffin Wax</td>
<td>Lamb</td>
</tr>
<tr>
<td>Potassium Dihydrogen Orthophosphate</td>
<td>Sigma</td>
</tr>
<tr>
<td>Practamount</td>
<td>ASCO Laboratories</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>BDH</td>
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<td>Sodium Citrate</td>
<td>Sigma</td>
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<td>Tris (Hydroxymethyl) Methylamine</td>
<td>BDH</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>BDH</td>
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<tr>
<td>TritonX-100</td>
<td>Sigma</td>
</tr>
<tr>
<td>Xylene</td>
<td>BDH</td>
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APPENDIX II - Constituents and Preparation of Buffers and Solutions

Neutral Buffered Formalin
1. Dissolve 510mg disodium hydrogen orthophosphate, 325mg potassium dihydrogen orthophosphate and 9g sodium chloride in 900ml ddH2O.
2. Add 100ml formaldehyde.
3. Adjust to pH 6.8 using hydrochloric acid.

3-Aminopropyltriethoxysilane Coated Slides
1. Wash slides in 1% extran (v/v in ddH2O) for 1 hour.
2. Rinse for 2 hours in hot running water.
3. Air dry.
4. Place in 1% 3-aminopropyltriethoxysilane (v/v in acetone) for 5 minutes.
5. Rinse slides in acetone for 5 minutes.
6. Wash in running tap water for 5 minutes.
7. Dry at room temperature overnight or 60°C for 1 hour

10x Tris Buffered Saline (pH 7.6 at 25°C)
1. Dissolve 87.6g sodium chloride, 60.6g Tris-HCl, and 13.9 g Tris-base in 1 litre ddH2O.
2. Adjust pH to 7.6

0.1% α-Chymotrypsin Solution
1. Place sections in pre-warmed Tris buffered saline at 37°C for 5 minutes.
2. Place in solution containing 300ml Tris buffered saline, 30mg α-chymotrypsin, and 300mg calcium chloride at 37°C for 20 minutes.
3. Rinse slides with Tris buffered saline.

Sodium Citrate Solution (Pressure Cooker)
1. Dissolve 5.88g of tri-sodium citrate solution in 1965ml of distilled water
2. Add 44ml of 0.2M hydrochloric acid solution
3. Adjust pH to 6.0
4. Bring to the boil in a pressure cooker
5. Add slides in a metal rack and place lid on pressure cooker and bring to pressure
6. Time for 3 minutes
7. Run running tap water over the pressure cooker
8. Release pressure, remove lid, and run water into the pressure cooker for 10 minutes.
9. Remove slides and place in Tris buffered saline for 5 minutes

**0.2% Triton-X 100**

1. Place sections in pre-warmed Tris buffered saline at $37^\circ C$ for 5 minutes.
2. Place in solution containing 300ml Tris buffered saline and 600μl Triton-X-100 at $37^\circ C$ for 30 minutes.
3. Rinse slides with Tris buffered saline.

**ABComplex/Alkaline Phosphatase Kit**

1. Mix 1 drop avidin and 1 drop biotin with 5ml ddH2O.
2. Vortex and leave for at least 30 minutes before use.

**Substrate Solution For Phosphatase Staining (Imunohistochemistry)**

1. Dissolve 1 Tris buffered saline tablet and 1 Fast red tablet in 10ml ddH2O.
2. Filter through a 0.2μm filter.
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VEGF localisation in diabetic retinopathy

Mike Boulton, David Foreman, Gillian Williams, David McLeod

Abstract

Aim—To determine the staining pattern of vascular endothelial growth factor (VEGF) at different stages of diabetic retinopathy (including post-laser photocoagulation) and to compare staining in excised fibrovascular and fibrocellular (non-diabetic) preretal membranes.

Methods—Immunohistochemical localisation of VEGF, using antibodies raised against VEGFα, and VEGFβ, was carried out on specimens of normal human retina (n=15), diabetic retinas ((a) with no overt retinopathy (n=19), (b) with intraretinal vascular abnormalities but no proliferative retinopathy (n=6), (c) with active proliferative retinopathy (n=6), (d) with no residual proliferative retinopathy after photocoagulation therapy (n=15), excised diabetic fibrovascular membranes (n=19), and non-diabetic fibrocellular membranes (n=7). The degree and pattern of immunostaining was recorded.

Results—In general, VEGF was absent from the majority of normal retinas. VEGF staining was apparent in most diabetic tissues but the staining pattern was dependent on both the specificity of the antibody used and the category of tissue. Staining with the VEGFα antibody was generally confined to endothelial cells and perivascular regions while the VEGFβ antibody was also associated with extravascular components of the inner retina. Intensity of immunostaining in diabetic retinas which had undergone laser surgery for proliferative retinopathy was reduced to basal levels. Moderate to intense immunostaining was observed in all fibrovascular and fibrocellular membranes examined.

Conclusions—This study supports a circumstantial role for VEGF in the pathogenesis of both the preclinical and proliferative stages of diabetic retinopathy.

Materials and methods

DONOR EYES

Donor human eyes, fixed in 10% neutral buffered formalin (NBF) within 12 hours post mortem, were provided by the National Disease Research Interchange (NDRI), Philadelphia, USA. The anterior segment was removed and biomicroscopy of the posterior segment performed (a) to note overt features of retinopathy (for example, preretinal membranes, cotton wool spots, haemorrhages) and (b) to determine the extent of any scatter photocoagulation. Eyes were categorised as follows:

Normal—15 human eyes with no known ophthalmic disease and no history of diabetes.
The posterior segment of each eye was cut in the sagittal plane through the centre of the optic nerve head. Cuts were then made perpendicular to this line (a) on the horizontal midline on the nasal side and (b) two cuts were made approximately 5 mm above and below the midline on the temporal side. A final vertical cut was made parallel to the initial cut and approximately 3 mm lateral to the macula. May PM sections (1-1.3 cm in length in the case of retinal specimens) were cut and fixed in 10% NBF immediately upon removal for a minimum of 12 hours before paraffin wax embedding.

IMMUNOHISTOCHEMISTRY

Five μm sections (1-1.3 cm in length) were cut and mounted on APES-coated slides. Deparaffinised sections were digested with 0.01% chymotrypsin for 20 minutes at 37°C. The sections were incubated in 10% milk proteins (Marvel)/10% normal rabbit serum (Dako) in TBS for 60 minutes at room temperature. Excess blocking solution was removed and the sections incubated overnight at 4°C in either (a) polyclonal goat anti-human VEGF (R&D Systems) raised against purified insect cell line S/21 derived recombinant human VEGF165 or (b) polyclonal goat anti-human VEGF 121,165,189 (Santa Cruz Biotechnology) raised against a peptide corresponding to amino acids 1-20 mapping at the amino terminus of VEGF of human origin and which recognises the 121, 165, and 189 amino acid splice variants of VEGF; both were diluted in TBS to 5 μg/ml. Following two 3 minute washes, the sections were incubated with biotinylated rabbit anti-goat IgG (Dako) diluted to 1/600 in TBS for 30 minutes at room temperature. The sections were subsequently incubated with an avidin-biotin alkaline phosphatase reaction complex (Dako) and antibody binding visualised by incubation in fast red substrate solution (Sigma) resulting in the formation of a red product. Immunostained sections were counterstained with haematoxylin.

CONTROLS FOR IMMUNOSTAINING

Negative controls included (1) omission of the primary antibody, (2) substitution of the primary antibody with an inappropriate goat antibody (goat anti-human colostrum whey (Sigma) at the same concentration as the primary antibody), and (3) incubation of 100 μl (0.5 μg) of anti-VEGF 165 antibody with 1 μg of recombinant human VEGF 121,165 (R&D Systems) overnight at 4°C before use in the above protocol.

ASSESSMENT OF IMMUNOSTAINING

The degree and pattern of immunostaining both within and between specimens as observed by standard light microscopy was assessed and recorded by two independent observers but pathological status of the specimen was obvious. The intensity of staining was graded qualitatively as background (corresponding to the level of staining seen in the negative controls), weak, moderate, or intense (corresponding to the highest level of immunoreactivity observed). These intensities were recorded as 0, 1, 2, and 3 respectively. For each retinal specimen staining intensity was recorded for the choroid, RPE, photoreceptor inner and outer segments, outer nuclear layer, inner nuclear layer, retinal vessels, ganglion cell layer, and internal limiting membrane. For

Donor age ranged from 20 to 92 years (mean 56 years).

Diabetic with no overt retinopathy—19 human eyes from diabetics with no clinical history of PDR and no overt features of retinopathy or retinal photocoagulation. Donor age ranged from 37 to 76 years (mean 58 years). Duration of diabetes ranged from 3 to 18 years (mean 9 years).

Diabetic with PDR—six human eyes from diabetics defined clinically as having PDR and exhibiting preretinal membranes. Donor age ranged from 20 to 41 years (mean 29 years). Duration of diabetes ranged from 3 to 18 years (mean 11 years). Donors were diabetic with PDR and having received scatter laser photocoagulation (no details were available as to time post laser). No preretinal membranes were observed. Donor age ranged from 41 to 82 years (mean 63 years). Duration of diabetes ranged from 3 to 35 years (mean 17 years).

METHODS

The posterior segment of each eye was cut in the sagittal plane through the centre of the optic nerve head. Cuts were then made perpendicular to this line (a) on the horizontal midline on the nasal side and (b) two cuts were made approximately 5 mm above and below the midline on the temporal side. A final vertical cut was made parallel to the initial cut and approximately 3 mm lateral to the macula. Five μm sections were cut from a portion of retina/choroid/sclera (a) approximately 3 mm lateral to the macula and perpendicular to the horizontal plane (this region was chosen owing to its susceptibility to retinal changes associated with diabetes) and (b) other representative areas across the retina (for example, areas of neovascularisation).

FIBROVASCULAR MEMBRANES

Nineteen fibrovascular preretinal membranes were obtained from 19 eyes during closed microsurgery for sequelae of PDR at the Manchester Royal Eye Hospital. Membranes were fixed in 10% NBF immediately upon removal for a minimum of 12 hours before paraffin wax embedding.

FIBROCELLULAR MEMBRANES

Seven non-vascularised epiretinal membranes were obtained from seven eyes of non-diabetic patients during closed microsurgery for elimination of retinal traction at the Manchester Royal Eye Hospital. Membranes were fixed in 10% NBF immediately upon removal for a minimum of 12 hours before paraffin wax embedding.

IMMUNOHISTOCHEMISTRY

Five μm sections (1-1.3 cm in length) were cut and mounted on APES-coated slides. Deparaffinised sections were digested with 0.01% chymotrypsin for 20 minutes at 37°C. The sections were incubated in 10% milk proteins (Marvel)/10% normal rabbit serum (Dako) in TBS for 60 minutes at room temperature. Excess blocking solution was removed and the sections incubated overnight at 4°C in either (a) polyclonal goat anti-human VEGF165 (R&D Systems) raised against purified insect cell line S/21 derived recombinant human VEGF165 or (b) polyclonal goat anti-human VEGF 121,165,189 (Santa Cruz Biotechnology) raised against a peptide corresponding to amino acids 1-20 mapping at the amino terminus of VEGF of human origin and which recognises the 121, 165, and 189 amino acid splice variants of VEGF; both were diluted in TBS to 5 μg/ml. Following two 3 minute washes, the sections were incubated with biotinylated rabbit anti-goat IgG (Dako) diluted to 1/600 in TBS for 30 minutes at room temperature. The sections were subsequently incubated with an avidin-biotin alkaline phosphatase reaction complex (Dako) and antibody binding visualised by incubation in fast red substrate solution (Sigma) resulting in the formation of a red product. Immunostained sections were counterstained with haematoxylin.

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ASSESSMENT OF IMMUNOSTAINING

The degree and pattern of immunostaining both within and between specimens as observed by standard light microscopy was assessed and recorded by two independent observers but pathological status of the specimen was obvious. The intensity of staining was graded qualitatively as background (corresponding to the level of staining seen in the negative controls), weak, moderate, or intense (corresponding to the highest level of immunoreactivity observed). These intensities were recorded as 0, 1, 2, and 3 respectively. For each retinal specimen staining intensity was recorded for the choroid, RPE, photoreceptor inner and outer segments, outer nuclear layer, inner nuclear layer, retinal vessels, ganglion cell layer, and internal limiting membrane. For
VEGF localisation in diabetic retinopathy

**Results**

VEGF staining was apparent in most diabetic tissue but the staining pattern was dependent on both the specificity of the antibody used and the category of tissue (see Figs 1 and 2 and Tables 1 and 2). Staining with the anti-VEGF antibody generally confined to endothelial cells (whether retinal or choroidal) and perivascular regions whereas staining with the VEGF antibody was also associated with non-vascular components of the inner retina. No correlation was found between staining intensity/distribution and either donor age, postmortem time, or duration of diabetes (where known).

**NORMAL**

Immunostaining with the anti-VEGF antibody was absent in the majority of retinas examined while retinal staining with the anti-VEGF antibody, although generally absent, did result in weak staining associated with retinal vessels (7/15) and in the RPE (3/15) (Fig 1A; Tables 1 and 2). Weak to moderate staining with the anti-VEGF antibody was also observed in the choroidal vessels (12/15). Staining was absent from the inner and outer retina in all but one specimen.

**DIABETIC WITH NO OVERT RETINOPATHY**

Staining intensity with the anti-VEGF antibody was not elevated above that observed in non-diabetic retinas. Twelve of 19 specimens exhibited some positive staining of endothelial cells of inner retinal vessels, while 9/19 were positive in vessel basement membranes (generally those diabetic retinas with thickened endothelial basement membranes). By contrast, immunostaining with the anti-VEGF antibody, albeit weak to moderate, was elevated in all tissue layers examined compared with that observed in normal retinas (Fig 1B; Table 2). Immunostaining was most intense in the inner retinal and ganglion cell layers (13/19).

**DIABETIC WITH OBVIOUS INTRARETINAL VASCULAR CHANGES BUT NO EVIDENCE OF PDR**

Intense immunostaining with the anti-VEGF antibody of the inner retinal vessels was seen in all diabetic retinas (6/6) with active neovascular PDR membranes on their surfaces (Fig 1E). Equally intense staining was observed within the membranes themselves (Fig 1E). While immunoreactivity for VEGF was found around choroidal vessels in all these PDR retinas, with staining ranging from weak to intense, the RPE layer and outer retina were generally VEGF negative. The immunostaining pattern with the anti-VEGF antibody showed a similar moderate to high intensity staining of retinal vessels but, in addition, there was increased staining in the outer nuclear layer, inner retina, ganglion cell layer, and internal limiting membrane (ILM) compared with the other tissue groups (Fig 1F).

### Table 1 Mean (SD) intensity of immunostaining of the retina/choroid using an anti-VEGF antibody

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<th>Tissue layer</th>
<th>Choroidal vessels</th>
<th>RPE</th>
<th>Outer retina</th>
<th>Inner retina</th>
<th>Retinal vessels</th>
<th>Ganglion cell layer</th>
<th>ILM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n=12)</td>
<td>1.6 (0.3)</td>
<td>0.3 (0.2)</td>
<td>0</td>
<td>0.3 (0.2)</td>
<td>0.9 (0.2)</td>
<td>0.1 (0.1)</td>
<td>0</td>
</tr>
<tr>
<td>Diabetic</td>
<td>1.6 (0.2)</td>
<td>0.1 (0.1)</td>
<td>0</td>
<td>0.4 (0.2)</td>
<td>1.2 (0.2)</td>
<td>0.2 (0.1)</td>
<td>0.1 (0.1)</td>
</tr>
<tr>
<td>No overt retinopathy (n=19)</td>
<td>2.3 (0.3)</td>
<td>0.5 (0.3)</td>
<td>0.2 (0.2)</td>
<td>0.3 (0.2)</td>
<td>2.2 (0.5)</td>
<td>1.0 (0.4)</td>
<td>0.3 (0.2)</td>
</tr>
<tr>
<td>PDR (n=6)</td>
<td>2.2 (0.4)</td>
<td>0.4 (0.3)</td>
<td>0.6 (0.2)</td>
<td>0.6 (0.2)</td>
<td>3.0 (0.9)</td>
<td>0.6 (0.2)</td>
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</tr>
<tr>
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<td>0.2 (0.1)</td>
<td>0.1 (0.1)</td>
</tr>
</tbody>
</table>

0 = background, 1 = weak, 2 = moderate, 3 = intense.

### Table 2 Mean (SD) intensity of immunostaining of the retina/choroid using an anti-VEGF antibody

<table>
<thead>
<tr>
<th>Tissue layer</th>
<th>Choroidal vessels</th>
<th>RPE</th>
<th>Outer retina</th>
<th>Inner retina</th>
<th>Retinal vessels</th>
<th>Ganglion cell layer</th>
<th>ILM</th>
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<td>Diabetic</td>
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<td>1.0 (0.2)</td>
<td>1.9 (0.2)</td>
<td>1.5 (0.2)</td>
</tr>
<tr>
<td>No overt retinopathy (n=19)</td>
<td>0.5 (0.2)</td>
<td>0.8 (0.4)</td>
<td>0.3 (0.3)</td>
<td>1.2 (0.5)</td>
<td>1.1 (0.4)</td>
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<td>1.3 (0.4)</td>
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<tr>
<td>Vascular abnormalities (n=6)</td>
<td>1.0 (0.5)</td>
<td>1.5 (0.4)</td>
<td>1.8 (0.3)</td>
<td>2.2 (0.3)</td>
<td>2.0 (0.4)</td>
<td>1.8 (0.4)</td>
<td></td>
</tr>
<tr>
<td>Laser, no PDR (n=15)</td>
<td>0.7 (0.2)</td>
<td>1.2 (0.3)</td>
<td>1.2 (0.3)</td>
<td>1.1 (0.2)</td>
<td>1.0 (0.2)</td>
<td>1.2 (0.2)</td>
<td>1.0 (0.2)</td>
</tr>
</tbody>
</table>

0 = background, 1 = weak, 2 = moderate, 3 = intense.
Figure 2 Photomicrographs demonstrating VEGF immunostaining of PDR retina and excised membranes. Intense immunostaining for VEGF is localised to the vasculature (A) while VEGF immunostaining is observed in both vascular and extravascular tissue (B). Moderate to intensive staining can be observed in all specimens of excised fibrovascular (C) and fibrovascular (D) epiretinal membranes. Immunoreactivity for VEGF was abolished in control sections of PDR retina and membranes processed with omission of the primary antibody (E) or prior incubation of the antibody with VEGF (Magnification, A, B $\times$200; C, F $\times$70).

No correlation was observed between VEGF positive glial staining and active neovascularisation.

DIABETIC WITH SCATTER LASER PHOTOCOAGULATION BUT NO EVIDENCE OF PDR

The intensity of immunostaining in diabetic retinas that had undergone apparently successful laser therapy (that is, those with no preretinal neovascularisation) was similar to that observed for diabetic retinas with no overt retinopathy (Fig 1G, H). This was true for both antibodies used. In many laser treated retinas (11/15) a total absence of immunoreactivity was observed within inner retinal vessels, even those with thickened basement membranes. This was especially apparent in those vessels located near laser burns. Staining...
intensity was also reduced/absent in the outer nuclear layer, inner retina, ganglion cell layer, and ILM compared with diabetic retinas with PDR.

**FIBROVASCULAR MEMBRANES**

A similar staining pattern was observed for both anti-VEGF antibodies used. In general, two immunostaining profiles for VEGF were observed in the preretinal diabetic membranes; those in which staining was essentially confined to the vascular component (9/19) and those in which moderate to intense staining was also found in areas of acellular matrix (10/19) (Fig 2B, C). Staining of vessels was variable both between and within specimens, with those vessels at the periphery generally staining more intensely than those in the centre of the membrane. In addition, staining of extracellular matrix was variable but also tended to be highest at the periphery. Staining of non-vascular cells was weak and variable, being present in only 7/19 membranes.

**FIBROCELLULAR MEMBRANES**

VEGF staining was observed in all seven fibrocellular membranes; the staining profile was similar for both anti-VEGF antibodies. Staining was associated with pigmented cells, non-pigmented cells and surrounding matrix (Fig 2D). The intensity of staining was highly variable both within and between membranes and ranged from weak to intense. Intensity of staining in the matrix was generally greater than that found in the cellular areas.

**CONTROLS**

Immunoreactivity for VEGF was abolished in sections processed with omission or substitution of the primary antibody (Fig 2E) and considerably reduced by prior incubation of the antibody with recombinant VEGF165 (Fig 2F).

**Discussion**

We have demonstrated changes in the profile and intensity of VEGF staining dependent upon the severity of diabetic retinopathy. The most intense staining correlated with active neovascularisation and was present in both preretinal vessels and associated inner retinal vessels. Interestingly, the elevation of immunostaining for VEGF in diabetic retinas with no overt retinopathy infers that VEGF may play a role in both the preclinical and the proliferative stages of diabetic retinopathy.

Lutty and co-workers detected VEGF immunoreactivity within some smooth muscle cells of retinal arterioles and in the pericytes and some endothelial cells of retinal capillaries in non-diabetic human retinas. Furthermore, they noted a significantly increased immunoreactivity in the contractile elements of retinal vessels, in the endothelium of human diabetic retina and in and around the choroidal vessels, results consistent with those presented here. Immunohistochemical staining for VEGF has also been shown to be markedly increased in the retina of streptozotocin treated diabetic rats, with positive staining located in and around the inner retinal capillaries. However, in areas of non-perfused human diabetic retina and in rabbit and primate models of ocular angiogenesis, in situ hybridisation studies have shown hypoxia induced VEGF expression not in the component cells of retinal vessels but in the inner and outer nuclear layers or the ganglion cell layers. This distribution is in agreement with immunostaining of VEGF within Muller cells and in the ganglion cell layer in oxygen induced neovascularisation models in rat retina and in diabetic retina. We could only observe significant staining in these extravascular regions with one of the anti-VEGF antibodies used—that is, VEGF(165,189). This difference in specificity may reflect either (a) the availability of the appropriate epitope or (b) differential expression of VEGF isoforms within the retina. The generation of antibodies against the 121 and 189 VEGF isoforms may help address this question.

Rather than defining the sites of potential VEGF synthesis, the increase in staining for VEGF protein in diabetic retinal vessels demonstrated in our study, especially in eyes with neovascularisation, may reflect the localisation of sites of action or accumulation of the factor. Since VEGF binds to hypoxia inducible receptors found on endothelial cells, such cells surrounding non-perfused areas of diabetic inner retina may be likely to manifest increased VEGF binding. In addition, VEGF, especially the larger splice variants (VEGF(165,189)), binds to heparin and increased staining and around retinal vessels may to some extent reflect binding to heparan sulphate associated with basement membrane thickening in diabetes.

We have demonstrated that VEGF protein increases in diabetic eyes and that staining is greatest in PDR. The role of VEGF in preclinical retinopathy is unclear but may relate to vascular permeability. Raised VEGF levels are known to raise vascular permeability, a feature prominent in background and preproliferative diabetic retinopathy. These observations suggest that VEGF is elevated in the diabetic retina in the absence of hypoxia and extensive retinal ischaemia inferring that other mechanisms (for example, hyperglycaemia) may upregulate VEGF. However, it should be noted that in this, as in other, studies it is difficult to determine the degree of retinal ischaemia, if any, in postmortem diabetic retinas with preproliferative retinopathy. While vascular permeability has been suggested as being a critical step in angiogenesis, VEGF itself is also likely to directly affect other cellular events associated with neovascularisation. These observations are supported by Tolentino et al who reported that intravitreal injection of VEGF into primates resulted in leaky vessels, progressively dilated and tortuous vessels, microaneurysms, haemorrhage, and capillary closure. Our observation that high levels of VEGF are associated with the periphery of preretinal vessels is further support that VEGF plays an
VEGF localisation in diabetic retinopathy

The progression of preretinal neovascularisation. The intense staining of acellular matrix in many of the membranes may also reflect the matrix binding properties of VEGF. VEGF present in PDR membranes may derive from the retina but may also be synthesised by the cells within the membrane. We observed staining for VEGF within non-vascular cells in some membranes, while mRNA for VEGF has been found in diabetic membranes. Vitreous fluid from patients with active retinal neovascularisation has previously been shown to contain high concentrations of VEGF. Independent of its origin, membrane and vitreous associated VEGF may propel the neovascular response resulting in membrane growth.

VEGF immunoreactivity in epiretinal membranes is in agreement with Chen and colleagues. It is difficult to explain the presence of VEGF but it may reflect the hypoxic nature of this avascular tissue or that VEGF is modulating non-vascular cells; RPE cells and fibrocellular membranes are known to express VEGF receptors.

Scatter laser photocogulation induces re-gression of active diabetic neovascularisation. We have shown that immunostaining for VEGF is reduced in diabetic retinas that have no overt preretinal neovascularisation following laser therapy. This is in close agreement with Aiello et al who found decreased vitreous levels of VEGF in patients after laser therapy. It is possible that a reduction in retinal ischaemia after laser treatment reduces the production of VEGF suppressing neovascularisation and leading to regression and quiescence.

An expanding body of evidence, to which these results contribute, now suggests that VEGF may be Michaelson's ischaemia-induced ocular angiogenic factor. However, it is unlikely that VEGF functions in isolation. Other angiogenic factor levels have been found to be elevated in diabetic retina, fibrovascular membranes, and vitreous including bFGF, IGF-1, TGF-β, PDGF, and placenta growth factor (PIGF). Thus, VEGF may work synergistically with other factors; repeated intravitreal injection of VEGF into primates produces vascular abnormalities associated only with background retinopathy inferring that additional growth factors are required to initiate preretinal neovascularisation. Furthermore, VEGF may play a largely ignored, but important, role in modifying vascular permeability during the early stages of preproliferative retinopathy. Studies which determine the biological effects of VEGF and its interactions with other growth factors should increase our understanding of the vasoproliferative retinopathies and provide opportunities for new therapeutic interventions for these blinding conditions.

This work was supported by the British Diabetic Association, the Guide Dogs for the Blind Association, the Wellcome Trust, and the Manchester Royal Eye Hospital Endowment Fund.


2 Michaelson IC. The mode of development of the vascular system in the retina with some observations on its significance for certain retinal disorders. Trans Ophthalmol Soc UK 1948;68:137-80.


Immunolocalisation of the VEGF receptors FLT-1, KDR, and FLT-4 in diabetic retinopathy

Gillian Smith, David McLeod, David Foreman, Mike Boulton

Abstract

Aim—To determine the spatial and temporal changes in the staining pattern of the VEGF receptors FLT-1, KDR, and the putative receptor FLT-4 during the pathogenesis of diabetic retinopathy.

Methods—Immunohistochemical localisation of VEGF receptors, using antibodies against FLT-1, FLT-4, and KDR, was carried out on specimens of normal human retina (n=10), diabetic retinas (a) with no overt retinopathy (n=12), (b) with intraretinal vascular abnormalities but no proliferative retinopathy (n=5), (c) with active proliferative retinopathy (n=6), and (d) with no residual proliferative retinopathy after scatter photocoagulation therapy (n=14), and surgically excised diabetic fibrovascular membranes (n=11).

The degree and pattern of immunostaining was recorded.

Results—FLT-1 staining was apparent in the retinas from both non-diabetic and diabetic retinas; weak to moderate staining was generally confined to the inner nuclear layer, the ganglion cell layer, and the retinal vessels during all stages of the disease process. Staining of the retinal vessels was raised in diabetic tissue compared with non-diabetic tissue. The pre-retinal vessels of the diabetic subjects stained moderately to intensely for FLT-1. In contrast with FLT-1 staining minimal immunostaining for KDR was demonstrated in the non-diabetic eyes and the unilatered eyes; however, weak staining for KDR was observed in the inner nuclear layer and the ganglion cell layer of the unilatered eyes with diabetic changes. In those retinas with preretinal neovascularisation KDR immunoreactivity was moderate to intense in the intra- and pre-retinal vessels. However, in the excised membranes, where the vessels may have been in a quiescent state, the levels of KDR were weak to moderate. After apparently successful laser treatment KDR staining was reduced in the intraretinal vessels. Minimal FLT-4 staining was observed throughout normal eyes while weak to moderate FLT-4 staining was generally confined to the inner nuclear layer and the ganglion cell layer of the unilatered diabetic eyes. Weak to moderate levels of FLT-4 staining were observed in the intraretinal vessels except after apparently successful laser treatment where reduced levels of staining were observed. Weak to moderate staining was observed in the preretal vessels.

Conclusions—This study supports a role for FLT-1, KDR, and possibly FLT-4 in the pathogenesis of diabetic retinopathy; however, their specific roles in the progression of the disease may differ.

Proliferative diabetic retinopathy (PDR), the archetypical vasoproliferative retinopathy (VPR), is characterised by preretinal neovascularisation and fibrosis, ultimately leading to vitreous haemorrhage and traction retinal detachment. A number of growth factors have been implicated in PDR of which vascular endothelial growth factor (VEGF) is considered to be of major importance since (a) it is a diffusible factor, (b) it increases vascular permeability, (c) it modulates angiogenesis, (d) it stimulates endothelial cell proliferation and migration, (e) it is upregulated in response to hypoxia, and (f) agents which inhibit the binding of VEGF to its receptors have been demonstrated to reduce neovascularisation. In situ hybridisation, northern blotting, and immunohistochemistry have demonstrated increased expression of VEGF in animal models for VPRs and in diabetic human retina.

VEGF is believed to act through high affinity receptors located on endothelial cells. These receptors are autophosphorylating type III tyrosine kinases and consist of KDR (FLK-1 in mouse, TKR-C in rats, Quek1 and 2 putative avian) and FLT-1 receptors. Both receptors are characterised by the presence of seven immunoglobulin-like domains in their extracellular region and are expressed during embryogenesis where they appear to play an important role in endothelial growth and differentiation during vasculogenesis and angiogenesis. FLT-1 is believed to regulate metabolic activity including vascular permeability while KDR is considered to modulate angiogenic responses (for example, endothelial cell migration and proliferation). The importance of FLT-1 is further inferred by the recent demonstration that placenta growth factor (PIGF) is associated with diabetic retinopathy. PIGF acts through the FLT-1 receptor. A third tyrosine kinase receptor may be important in VEGF recognition by endothelial cells; FLT-4, which has a similar structure to FLT-1 and KDR, is expressed in the placenta and in several mouse tissues during embryogenesis.

Although there are a large number of reports documenting upregulation of VEGF mRNA
and protein in the VPRs there is very little information on the profile of VEGF receptors. In this study we used immunohistochemistry to detect FLT-1, KDR, and FLT-4 protein in (a) normal human retinas, (b) diabetic retinas with various stages of retinopathy, and (c) in preretinal fibrovascular membranes excised during diabetic vitrectomy.

Materials and methods

DONOR EYES

A total of 47 eyes enucleated and fixed in 10% neutral buffered formalin, within 10 hours post mortem, were obtained from the National Disease Research Interchange (NDRI), Philadelphia, USA. Each eye was dissected into an anterior and posterior segment. A complete medical history was not available for all donors but details were available regarding glycaemic management. Of the 37 diabetic donors 25 had been injecting insulin for at least 6 months (mean age 62 years) and 12 were not receiving insulin treatment but did use oral hypoglycaemic drugs (mean age 65 years). Examination of the posterior segment was performed by an ophthalmologist (DM) using a Zeiss Stemi SV8 zoom dissecting microscope with Schott light source (a) to note overt features of retinopathy (for example, the presence of preretinal membranes, cotton wool spots, microaneurysms,

Figure 1 Photomicrographs demonstrating FLT-1 immunostaining of non-diabetic retina (A), diabetic retina with no obvious retinopathy (B), diabetic retina with obvious preretinal vascular changes but no evidence of PDR (C), the same retina stained with GFAP (D), diabetic retina with PDR (E, F, G), diabetic retina post laser but with no residual PDR (H). Immunostaining for FLT-1 was greatest in the diabetic tissue compared with non-diabetic tissue. Increased staining was generally confined to the inner nuclear layer and the ganglion cell layer. Magnification A, G x94; B, C, E x118; D x156; F x378; H x236.
etc) and (b) to determine the extent of any scatter photocoagulation.

Eyes were categorised as follows:

Normal—10 human eyes with no known ophthalmic disease, no history of diabetes, and no abnormalities on biomicroscopy. Donors ranged in age from 34 to 89 years (mean 69 years).

Diabetic with no overt retinopathy—12 human eyes from diabetic donors with no clinical history and no overt biomicroscopic features of retinopathy or retinal photocoagulation. Donors ranged in age from 57 to 89 years (mean 77 years), five had been injecting insulin and seven had not. A complete medical history was unavailable for all donors but, in those where medical histories were known (10/12), the duration of diabetes was between 6 and 10 years (mean 7.3 years).

Diabetic with intraretinal changes but no evidence of PDR—five human eyes from diabetic donors with intraretinal changes on biomicroscopy but no clinical history or overt features of PDR or retinal photocoagulation. Retinas exhibited cotton wool spots and/or obvious microaneurysms or haemorrhages. Donors ranged in age from 62 to 96 years (mean 74 years), three had been injecting insulin and two had not. A complete medical history was known for four donors, the duration of diabetes being between 3 and 21 years (mean 13 years).

Diabetic with preretinal PDR—six human eyes from diabetic donors defined clinically as having PDR and exhibiting preretinal membranes when examined by biomicroscopy. All eyes had previously received laser photocoagulation. Donors ranged in age from 37 to 76 years (mean 58 years), all had been injecting insulin. Duration of diabetes ranged from 3–29 years (mean 14.3 years).

Diabetic with scatter laser photocoagulation but no evidence of residual PDR—14 human eyes from diabetic donors defined clinically as having had PDR and having received scatter laser photocoagulation. No preretal membranes could be observed when retinas were examined by biomicroscopy. Donors ranged in age from 40 to 82 years (mean 57 years), 11 had been injecting insulin and three had not. A complete medical history was known for 13 donors, the duration of diabetes being between 10 and 35 years (mean 20 years).

The posterior segment of each eye was cut in the sagittal plane through the centre of the optic nerve head. Cuts were then made perpendicular to this line (a) on the horizontal midline on the nasal side and (b) at approximately 5 mm above and below the midline on the temporal side. A final vertical cut was made parallel to the initial cut and approximately 3 mm temporal to the macula. For this study tissue was wax embedded and 5 μm sections were cut from a portion of retina/choroid/sclera (a) approximately 3 mm lateral to the macula and perpendicular to the horizontal plane (this region was chosen because of its susceptibility to retinal changes associated with diabetes) and (b) other representative areas across the retina (for example, areas of neovascularisation).

Fibrovascular membranes—11 fibrovascular preretinal membranes excised at vitreous surgery from eyes with PDR were obtained from the Manchester Royal Eye Hospital. Membranes were fixed in 10% neutral buffered formalin immediately upon removal and for a minimum of 12 hours before wax embedding.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was undertaken as previously described. The 5 μm sections were cut and mounted on APES (Sigma) coated slides. Sections were dewaxed and rehydrated. They were blocked for 60 minutes with 10% milk protein (Marvel)/normal goat serum (Sigma) before incubation overnight at 4°C

![Figure 2 Photomicrographs demonstrating FLT-1 immunostaining of an excised fibrovascular membrane (A). Immunoreactivity for FLT-1 was abolished in a control specimen of excised membrane processed with omission of the primary antibody (B) and GFAP staining of the same excised membrane (C). Intense staining was observed around the vessels in excised membranes. Magnification A, B, C x60.](image-url)
with either (a) a polyclonal rabbit antibody raised against a peptide corresponding to amino acids 1312–1328 mapping at the carboxy terminus of FLT of human cell origin and reacting with FLT of mouse, rat, and human cell origin (R&D Systems) diluted to 1 µg/ml in TRIS buffered saline (TBS), (b) a polyclonal rabbit antibody raised against a GST fusion protein containing FLK-1 sequences corresponding to amino acids 1158–1345 mapping at the carboxy terminal of FLK-1 of mouse origin (that is, the murine form of KDR) and reacting with FLK-1 of mouse, rat, and human cell origin (R&D Systems) diluted to 2 µg/ml in TBS, or (c) a polyclonal rabbit antibody raised against a peptide corresponding to amino acids 1279–1298 mapping at the carboxy terminus of FLT-4 of human origin and reacting with FLT-4 of human origin (R&D Systems) diluted to 1 µg/ml in TBS. A selection of slides were also stained for polyclonal rabbit antiglial fibrillary acidic protein (GFAP) antibody isolated from human spinal cord, directed against the 56 kD GFAP protein and reacting with GFAP of bovine, rat, and human origin (Euro-Diagnostica), diluted 1/50 in TBS. Negative controls were incubated with 0.2% goat serum in place of the primary antibody or substitution of the primary antibody with an inappropriate rabbit IgG at the same concentration as the primary antibody. Sections were washed three times with TBS and then incubated for 30 minutes with biotinylated goat anti-rabbit IgG (Sigma) and then incubated for 30 minutes with an avidin-biotin alkaline phosphatase reaction mixture (Dako Ltd). The sections were washed three times with TBS and then incubated with Fast Red TR/naphthol AS-MX substrate (Sigma). When the red colour had sufficiently developed the slides were washed in distilled water and counterstained with Mayer’s haematoxylin.

**ASSESSMENT OF IMMUNOSTAINING**

The degree and pattern of immunostaining both within and between specimens was assessed by standard light microscopy by two masked observers (both of whom obtained similar results). The intensity of staining was

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**Figure 3** Photographs demonstrating KDR immunostaining of non-diabetic retina (A), diabetic retina with PDR (B, C), the same retina stained with GFAP (D), and diabetic retina post laser but with no residual PDR (E). Immunostaining for KDR was greatest in the diabetic tissue with PDR and was minimal in most other diabetic tissue. Interestingly, immunostaining in the diabetic retina which had undergone apparently successful laser treatment was reduced compared with the staining intensity in the retina with PDR. Immunoreactivity for KDR was abolished in a control specimen of PDR retina processed with omission of the primary antibody (F). Magnification = A, C, D x156, B x60, E x118, F x78.
graded qualitatively as background (corresponding to the level of staining seen in the negative controls), weak, moderate, or intense (corresponding to the highest level of immunoreactivity), each of these being recorded as 0, 1, 2, and 3 respectively. For each retinal specimen staining intensity was recorded for choroid, RPE, photoreceptors, outer retina, inner retina, ganglion cell layer, and retinal vessels. For the fibrovascular membranes staining intensity was recorded for the vessels and the surrounding matrix. An average score was then calculated for each retinal layer within each group.

Results
Staining was observed in both non-diabetic and diabetic vascular and extravascular retinal tissue; increased immunostaining was observed in preretinal and intraretinal vessels of diabetic tissue compared with non-diabetic tissue (see Figs 1–4 and Tables 1–3). For all receptors variable staining of the vessels within each retina was observed with some vessels staining positive and some staining negative. In some instances staining was associated with both endothelial cells and the perivascular region of the vessels. The variability in staining within retinas of the same group did not show a correlation with donor age, the type of glycaemic control in the case of the diabetic groups, or time post mortem.

FLT-1 IMMUNOREACTIVITY
Staining intensity for FLT-1 was generally weak or absent in the choroidal vessels, the RPE, and the photoreceptors. Weak staining was observed in the outer nuclear layer of most tissue categories but staining intensity tended to be elevated in diabetic eyes with vascular abnormalities and in those which had been successfully lasered. Weak to moderate staining was observed in the inner nuclear layer and weak to intense staining was observed in the ganglion cell layer of all the tissue categories including the non-diabetic eyes (Fig 1A–C; E–H; Table 1). The pattern of staining in the ganglion cell layer appeared to be associated with the Müller cell feet as it co-localised with positive GFAP staining (Fig 1D). While weak staining was observed in the retinal vessels of the non-diabetic eyes and the diabetic eyes.

Figure 4  Photomicrographs demonstrating FLT-4 staining of normal retina (A), diabetic retina with obvious vascular intraretinal changes but no evidence of PDR (B), diabetic retina with PDR (C, D) and the same retina stained with GFAP (E). Immunostaining for FLT-4 was raised in diabetic tissue compared with non-diabetic tissue. Immunostaining was intermediate in the PDR specimens. Immunoreactivity for FLT-4 was abolished in a control specimen of PDR retina processed with omission of the primary antibody (F). Magnification A, D x156; B x118; C x94; E, F x60.
without vascular changes, staining was moderate in all the other categories of diabetic tissue (Fig 1A–C; E–H; Table 1). The highest intensity of FLT-1 staining in the intraretinal vessels was associated with successful laser treatment with most (13/14) retinas staining. In all tissue categories staining tended to be confined to small and venous vessels in the superficial layers, although in 5/14 lasered retinas and in 2/6 retinas with PDR (both of which had previously been lasered) staining of the arterial vessels was observed. The most intense staining for FLT-1 was observed in the vessels of preretinal membranes of the diabetic subjects who had PDR (Table 1). In this tissue category staining of the intraretinal vessels was associated both with the membranes and across the retina (Fig 1E, F, G). Staining was moderate in the excised membranes but staining tended to be confined to a proportion of the vessels within each membrane with 4/11 of the membranes demonstrating staining both around the vessels and in the adjacent matrix (Fig 2A). Weak staining was associated with the nonvascular components of the membranes and staining with GFAP antibody confirmed that some of the perivascular and extravascular staining was glial cell in origin (Fig 2C).

**KDR immunoreactivity**

KDR immunoreactivity was generally minimal or absent in the choroidal vessels, the RPE, and the retinal layers of all the categories (Fig 3A); however, weak staining was observed in the inner nuclear layer and the ganglion cell layer of the unlasered eyes with obvious diabetic changes. Minimal to weak staining of the retinal vessels was observed in most categories but it became moderate to intense in the retinal vessels of diabetics with PDR with all (6/6) of the retinas staining (Fig 3B, C; Table 2). In 4/6 diabetic retinas with PDR staining of the intraretinal vessels was associated with the membranes but in 2/4 of these staining was also observed in vessels across the retina. In all categories staining tended to be associated with small and venous vessels (with one exception which was a non-diabetic eye) and was always observed in the superficial retinal layers. Moderate staining of the preretinal vessels was observed in most of the membranes (Fig 3B, C). In some instances staining was observed in the perivascular region and extravascular region and staining with GFAP antibody confirmed this to be glial cell in origin (Fig 3D). In those retinas which had undergone apparently successful laser treatment staining was reduced (Fig 3E; Table 2). In the excised membranes staining tended to be confined to a proportion of the vessels within each membrane with 2/11 of the membranes demonstrating staining both around the vessels and in the adjacent matrix. Weak or absent staining was associated with the nonvascular components of the membranes and staining with GFAP antibody confirmed that some of the perivascular and extravascular staining was glial cell in origin.

### Table 1 Mean intensity (SD) of FLT-1 staining in the retina/choroid

<table>
<thead>
<tr>
<th>Condition</th>
<th>Choroid</th>
<th>RPE</th>
<th>Photo receptors</th>
<th>Outer nuclear layer</th>
<th>Inner nuclear layer</th>
<th>Ganglion cell layer</th>
<th>Retinal vessels</th>
<th>Membrane vessels</th>
<th>Membrane extravascular matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic (n=10)</td>
<td>0.70 (0.46)</td>
<td>1.10 (0.83)</td>
<td>0.60 (0.80)</td>
<td>0.20 (0.40)</td>
<td>1.10 (0.94)</td>
<td>1.50 (1.12)</td>
<td>1.0 (1.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No overt retinopathy (n=12)</td>
<td>0.92 (0.64)</td>
<td>0.58 (0.86)</td>
<td>0.42 (0.49)</td>
<td>0.33 (0.47)</td>
<td>1.42 (0.49)</td>
<td>1.92 (0.86)</td>
<td>1.25 (0.92)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intraretinal changes (n=5)</td>
<td>0.60 (0.80)</td>
<td>0.80 (0.75)</td>
<td>0.80 (1.17)</td>
<td>1.20 (1.17)</td>
<td>1.60 (0.80)</td>
<td>2.20 (0.75)</td>
<td>1.60 (1.02)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDR (n=6)</td>
<td>1.0 (0)</td>
<td>0.17 (0.37)</td>
<td>0 (0)</td>
<td>1.0 (0.82)</td>
<td>1.17 (0.69)</td>
<td>2.0 (0.82)</td>
<td>1.50 (1.12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laser, no residual PDR (n=14)</td>
<td>0.57 (0.49)</td>
<td>0.93 (1.05)</td>
<td>0.21 (0.56)</td>
<td>1.50 (0.65)</td>
<td>1.86 (0.74)</td>
<td>1.71 (0.45)</td>
<td>1.55 (0.78)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDR excised membranes (n=11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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0=background staining; 1=weak staining; 2=moderate staining; 3=intense staining.

### Table 2 Mean intensity (SD) of KDR staining in the retina/choroid

<table>
<thead>
<tr>
<th>Condition</th>
<th>Choroid</th>
<th>RPE</th>
<th>Photo receptors</th>
<th>Outer nuclear layer</th>
<th>Inner nuclear layer</th>
<th>Ganglion cell layer</th>
<th>Retinal vessels</th>
<th>Membrane vessels</th>
<th>Membrane extravascular matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic (n=10)</td>
<td>0.40 (0.49)</td>
<td>0.20 (0.40)</td>
<td>0.10 (0.30)</td>
<td>0.10 (0.30)</td>
<td>0.60 (0.66)</td>
<td>0.40 (0.49)</td>
<td>0.20 (0.40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No overt retinopathy (n=12)</td>
<td>0.08 (0.28)</td>
<td>0 (0)</td>
<td>0.25 (0.43)</td>
<td>0 (0)</td>
<td>0.75 (0.60)</td>
<td>0.75 (0.43)</td>
<td>0.75 (0.92)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intraretinal changes (n=5)</td>
<td>0.20 (0.40)</td>
<td>0 (0)</td>
<td>0.40 (0.80)</td>
<td>0 (0)</td>
<td>1.40 (1.20)</td>
<td>1.40 (0.80)</td>
<td>0.40 (0.69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDR (n=6)</td>
<td>0.67 (0.75)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.50 (1.12)</td>
<td>0.17 (0.37)</td>
<td>2.33 (0.75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laser, no residual PDR (n=14)</td>
<td>0.07 (0.26)</td>
<td>0.07 (0.24)</td>
<td>0.07 (0.26)</td>
<td>0 (0)</td>
<td>0.71 (0.45)</td>
<td>0.29 (0.45)</td>
<td>0.50 (0.82)</td>
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<tr>
<td>PDR excised membranes (n=11)</td>
<td></td>
<td></td>
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</table>

0=background staining; 1=weak staining; 2=moderate staining; 3=intense staining.

### Table 3 Mean intensity (SD) of FLT-4 staining in the retina/choroid

<table>
<thead>
<tr>
<th>Condition</th>
<th>Choroid</th>
<th>RPE</th>
<th>Photo receptors</th>
<th>Outer nuclear layer</th>
<th>Inner nuclear layer</th>
<th>Ganglion cell layer</th>
<th>Retinal vessels</th>
<th>Membrane vessels</th>
<th>Membrane extravascular matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic (n=10)</td>
<td>0.30 (0.46)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.10 (0.30)</td>
<td>0.90 (0.94)</td>
<td>0.50 (0.67)</td>
<td>0.70 (1.0)</td>
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</tr>
<tr>
<td>No overt retinopathy (n=12)</td>
<td>0.42 (0.49)</td>
<td>0.08 (0.28)</td>
<td>0.33 (0.75)</td>
<td>0 (0)</td>
<td>1.50 (0.76)</td>
<td>1.83 (0.80)</td>
<td>1.0 (0.71)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intraretinal changes (n=5)</td>
<td>0.20 (0.40)</td>
<td>0.20 (0.40)</td>
<td>0.66 (0.80)</td>
<td>0 (0)</td>
<td>1.80 (1.17)</td>
<td>2.0 (1.10)</td>
<td>1.20 (1.47)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDR (n=6)</td>
<td>1.0 (0.82)</td>
<td>0.17 (0.37)</td>
<td>0 (0)</td>
<td>0.33 (0.47)</td>
<td>1.0 (0.82)</td>
<td>1.50 (1.12)</td>
<td>1.50 (1.28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laser, no residual PDR (n=14)</td>
<td>0.57 (0.62)</td>
<td>0.29 (0.45)</td>
<td>0.21 (0.56)</td>
<td>0.21 (0.41)</td>
<td>0.80 (0.66)</td>
<td>0.79 (0.56)</td>
<td>0.93 (0.80)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDR excised membranes (n=11)</td>
<td></td>
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</tbody>
</table>

0=background staining; 1=weak staining; 2=moderate staining; 3=intense staining.
Diabetic retinas (Fig 4A) and diabetic retinas (Fig 4B, C, D). In the inner nuclear layer and the ganglion layer, FLT-4 immunoreactivity was only raised in the unlesioned eyes, after laser treatment the levels reduced (Table 3). Staining with GFAP antibody confirmed that FLT-4 staining was associated with glial cells of the retina (Fig 4E). In the retinal vessels, FLT-4 staining was low except in the PDR specimens where staining was weak to moderate (Fig 4C, D). In this tissue category staining in the intraretinal vessels was associated with the membranes in 3/6 retinas but staining in 2/3 of these was also observed in vessels across the retina. In all tissue categories staining tended to be associated with small and venous vessels of the superficial retinal layers although arterial staining was demonstrated in a small number of retina. Weak to moderate staining was also observed in the preretal vessels of the excised membranes. Staining tended to be associated with a proportion of vessels within each membrane with 2/11 of the membranes demonstrating staining both around the vessels and in the adjacent matrix. Minimal staining was associated with the non-vascular components of the membranes and staining with GFAP antibody confirmed that some of the perivascular and extravascular staining was glial cell in origin.

**Discussion**

The data presented in this study demonstrate (a) immunolocalisation of FLT-1, KDR, and FLT-4 receptors to retinal tissue and (b) upregulation of these receptors in diabetic retinopathy. These observations add further support for a role for VEGF family members in the initiation and progression of PDR.

Binding sites for VEGF have previously been demonstrated to be associated with vascular endothelial cells during the development of the vasculature,1-3,4-5 during pathological angiogenesis—for example, in healing wounds, in skin diseases, in hypersensitivity reactions, and in carcinomas,6-8. In vivo studies,9-10 These observations advance a regulatory role for VEGF and its receptors in angiogenesis occurring both during normal vascular development and in various pathologies.

The observation in this study that KDR is greatly elevated in both intra- and preretinal vessels in PDR tissue and minimal in normal retina and the quiescent vessels of lased diabetic retina with no evidence of PDR is in agreement with the view that KDR is involved in pathological angiogenesis. These findings correlate with the findings of various workers11-15 who reported high levels of VEGF in the vitreous of patients with active PDR. By contrast, FLT-1 was observed in both non-diabetic and diabetic vascular and avascular retinal tissue. The presence of FLT-1 in non-diabetic tissue may reflect its involvement in metabolic control—for example, control of vessel permeability and endothelial cell maintenance. Upregulation of FLT-1 in diabetic vessels, particularly those undergoing active neovascularisation, indicates that the receptor plays a role in PDR. Firstly, it may induce vascular leakage; FLT-1 is known to promote vascular permeability.16 Secondly, it has been suggested that it may participate in VEGF induced mitogenesis by heterodimer formation with KDR.17 Thirdly, FLT-1 may regulate VEGF induced angiogenesis; soluble form of FLT-1 can complex with the extracellular region of KDR and act as a negative regulator of VEGF action.18 Fourthly, PIGF which is associated with PDR acts through the FLT-1 receptor.19 FLT-4 represents a third putative receptor for the VEGF family which shares structural similarities with FLT-1 and KDR; it is believed to be a receptor for VEGF-C.20 FLT-4 immunolocalisation was minimal in non-diabetic eyes but was upregulated in diabetic tissue, especially in the inner nuclear layer, the ganglion cell layer, and intraretinal and preretinal vessels. These observations suggest that FLT-4 may have a role in the pathogenesis of diabetic retinopathy.

Several ocular cell types, in addition to vascular endothelial cells21-22 and pericytes,23 express VEGF receptors. VEGF receptors have been identified on cultured corneal cells,24 cultured lens epithelial cells,25 and cultured RPE cells.26-27 Increased levels of VEGF mRNA and protein have previously been demonstrated in retinal disorders in the cell bodies of the inner nuclear layer, the ganglion cell layer, and the outer nuclear layer.28-29 Studies on the developing retinal vasculature have also demonstrated VEGF mRNA and protein in the retinal glial cells.30-31 Chen and co-workers demonstrated intense VEGF staining in both vascular and extravascular epiretinal membranes.32 They also demonstrated FLT-1 but not KDR expression by glial cells in the epiretinal membranes and in cultured retinal glial cells. In our study we also demonstrated increased immunoreactivity for FLT-1, FLT-4, and, to a lesser extent, KDR in the intraretinal vessels of the retina which was particularly associated with the end feet of the Müller cells. Thus, these observations demonstrate that VEGF may act through its receptors via both autocrine and paracrine mechanisms. It may be that one of the functions of the retinal glial cells is as early detectors of the hypoxic environment occurring during the earlier stages of diabetic retinopathy. This could explain why in our study FLT-1, FLT-4, and to a lesser extent KDR, were associated with the glial cells before proliferation had occurred. These cells may respond to hypoxia by upregulating their receptors and secreting VEGF which acts on the endothelial cells. The sustained production of VEGF would eventually lead to an angiogenic response. Sustained production of VEGF may be maintained by a positive feedback mechanism to the receptors on the glial cells and the endothelial cells which could explain why increased levels of FLT-1 were observed in the glial cells of the eyes with PDR. An interesting observation was that in some of the membranes GFAP staining was observed both around the vessels and in the surrounding matrix which corresponded to the receptor immunoreactivity. It may be that these particular membranes were undergoing active...
neovascularisation or that there may have been hypoxic regions within these membranes.

VEGF receptor expression appears to be regulated by various stimuli including growth factor activity, hypoxia, as mentioned above, hypoxia. 1

In conclusion, this study confirms the presence of VEGF family receptors in the diabetic retina and indicates that while KDR appears to be involved principally with the angiogenic process (that is, PDR), FLT-1 may have a role in both normal endothelial cell homeostasis and in all stages of diabetic retinopathy. Therefore, any agent directed against VEGF or FLT-1 could have a detrimental effect on the normal structure and functioning of endothelial cells and vessels. A more attractive alternative would be to produce anti-VEGF or FLT-1 antibodies that could be targeted against KDR, a non-angiogenic molecule(s) with low toxicity directed against KDR. One study by Strawn and coworkers found that anti-angiogenesis compounds that can inhibit FLK-1/KDR tyrosine kinase activity as well as endothelial cell mitogenesis and blood vessel formation in the chorioallantoic membrane. Further studies are necessary to determine whether the receptors are active, to ascertain the stimulus for upregulation of the receptors, and to determine whether inhibition of receptor activation is the therapy of choice in preclinical angiogenesis.

This work was supported by the British Diabetic Association, The Guide Dogs for the Blind Association, and the Manchester Royal Eye Hospital Endowment Fund.


Rapid Publication

Loss of the Antiangiogenic Pigment Epithelium-Derived Factor in Patients With Angiogenic Eye Disease

Joachim Spranger,1,2 Martin Osterhoff,1,2 Manja Reimann,1,2 Matthias Möhlig,1,2 Michael Ristow,1,2 Mary Kay Francis,3 Vincent Cristofalo,3 Hans-Peter Hammes,4 Gillian Smith,5 Michael Boulton,5 and Andreas F.H. Pfeiffer1,2

Retinal neovascularization characterizes proliferative diabetic retinopathy (PDR). Pigment epithelium-derived factor (PEDF) has been shown to be a major antiangiogenic growth factor in the mammalian eye. PEDF expression is suppressed by hypoxia, and changes in PEDF have been correlated to the development of retinal neovascularization in animal models of hypoxic eye disease. However, whether this concept of a reduced angiogenesis inhibitor holds true in humans is as yet unclear. In this study, we analyzed the in vivo regulation of PEDF in patients with and without hypoxic eye disease. We used immunoblots to measure PEDF in ocular fluids obtained from 64 nondiabetic and diabetic patients. In addition, immunohistochemistry of PEDF was carried out in specimens of normal human retinas and retinas with various degrees of diabetic retinopathy. The PEDF concentrations in patients with PDR (P < 0.001) or extensive nondiabetic retinal neovascularization caused by retinal-vein occlusion (P < 0.001) were lower than in control patients. Levels of PEDF were replenished in PDR patients with previous retinal scatter photocoagulation compared with PDR patients without previous photocoagulation (P = 0.01). Immunohistochemistry revealed an interstitial staining pattern as expected for a secreted protein, with an intense staining in retinas of patients without proliferative eye disease. However, in patients with PDR, little or no staining was detectable. Our data strongly support the concept that retinal angiogenesis is induced by loss of the major angiogenesis inhibitor in the eye, PEDF, in combination with an increased expression of angiogenic growth factors such as vascular endothelial growth factor. Our findings suggest that substitution of angiogenesis inhibitors may be an effective approach in the treatment of PDR. Diabetes 50:2641-2645, 2001

The control of retinal angiogenesis is of critical importance for the preservation of vision. Retinal neovascularization characterizes proliferative diabetic retinopathy (PDR), which is still one of the most common causes of blindness worldwide. Retinal ischemia induces intracellular neovascularization, presumably by stimulating the expression of angiogenic growth factors and by inhibiting the release of antiangiogenic cytokines. Vitreal levels of angiogenic growth factors have been shown to be directly associated with the degree of retinal angiogenesis. The ability to monitor and grade retinal angiogenesis within the eye as well as the ability to aspirate vitreous, which is known to contain retina-derived growth factors in direct association to the stage of retinal angiogenesis, makes the eye an ideal setting in which to investigate the delicate balance of new vessel growth and the influence of specific growth factors in vivo in humans.

Pigment epithelium-derived factor (PEDF) protects cerebellar granule cells against neurotoxic agents and is also called early population doubling level cDNA-1 (EPC-1), reflecting its upregulation during cell cycle arrest (G0) in young but not in senescent cultured fibroblasts. Recent studies have shown that PEDF is a highly effective inhibitor of angiogenesis in animal and cell culture models. The production of PEDF was decreased by hypoxia, which is also a central pathogenic stimulus in PDR. Immune neutralization of PEDF diminished the ability of cadaveric human vitreous to inhibit migration of endothelial cells, thereby demonstrating that a loss of PEDF is functionally important in mediating angiogenic properties of human vitreous ex vivo. Most importantly, systemically administered PEDF prevented aberrant blood vessel growth in a murine model of ischemia-induced retinopathy. However, no information is yet available about the presence and regulation of PEDF in vivo in humans, particularly in hypoxia-induced proliferative retinopathy. If PEDF is involved in the control of retinal angiogenesis in humans, one would expect that PEDF is decreased in the ocular fluids of patients with hypoxia-induced proliferative retinopathy and that PEDF levels increase after at least
partially successful therapy, such as retinal photocoagulation. In this study, we attempted to ascertain whether intraocular concentrations of PEDF correlated with the state of retinal neovascularization by measuring PEDF in the ocular fluid of 64 patients. We also investigated whether retinal scatter photocoagulation is capable of replenishing PEDF in the ocular fluid of patients with PDR. Spatial and temporal changes in the expression of retinal PEDF were determined by immunohistochemical localization of PEDF in the human retinas of patients with different degrees of diabetic retinopathy.

RESEARCH DESIGN AND METHODS

Vitreous was obtained from 64 patients (32 women and 32 men). Patients without proliferative retinal disease (control subjects: n = 19, 6 women and 13 men, mean age 70 ± 3 years) were compared with patients with PDR (n = 37, 17 women and 20 men, mean age 61 ± 2 years, 6 patients with type 1 diabetes, 31 with type 2 diabetes, HbA1c 7.8 ± 0.6%) and patients with a nondiabetic neovascularizing eye disease caused by central-vein occlusion (Rubebiosis; n = 8, 2 women and 6 men, mean age 71 ± 3 years, no diabetes). A total of 27 patients with PDR had retinal photocoagulation before vitrectomy (PDR + previous retinal photocoagulation [PRP]), whereas 10 patients with PDR had no previous photocoagulation (PDR – PRP). PDR was considered to be active if there was extensive retinal neovascularization represented by perfused, multibranching preretinal capillaries and to be quiescent if mainly nonperfused or gliotic vessels were present. Altogether, 15 patients with PDR had active neovascularization, whereas 22 patients had quiescent retinal angiogenesis. A total of 13 patients with PDR + PRP had new vessels elsewhere (NVE), 3 had new vessels on the disk (NVD), and 11 had NVE + NVD. Age, HbA1c, and duration of diabetes did not differ significantly between patients with PDR + PRP and PDR – PRP (age 61 ± 4 and 61 ± 2 years, HbA1c 7.6 ± 0.3 and 8.2 ± 0.4%, duration of diabetes 18.3 ± 2 and 17 ± 4 years, respectively). Untreated samples of human vitreous were obtained during pars plana vitrectomy. Samples were aspirated under standardized conditions directly above the retina at the beginning of surgery and prepared as previously described (2). Ocular neovascular activity was determined by fluorescein photography, vitil lamp examination, or by the surgeon at the time of surgery.

Specimens for immunohistochemistry were obtained from the National Disease Research Interchange (NDRI), Philadelphia, Pennsylvania. Eyes were enucleated and fixed in 10% neutral buffered formalin within 10 h postmortem. Examination of the posterior segment was performed by an experienced ophthalmologist using a Zeiss Stemi SVH zoom dissecting microscope. Eyes were categorized as follows (n = 5 for each group): normal (A); diabetic without ocular abnormalities (B); diabetic with intraocular changes but no evidence of PDR (C); diabetic with PDR (D); and diabetic with scatter laser photocoagulation and no evidence of residual PDR (<E). Samples were prepared, and criteria for categorization were chosen as previously described (9).

Classification of specimens was performed before the experimental part of the study. The study was approved by the Ethical Committee of the University of Bochum, and informed consent was obtained from all patients included.

Western blot. PEDF was quantified by Western blotting using polyclonal PEDF-specific antibodies (anti-PEDF), which were raised as previously described (10). Blots were analyzed automatically by a digital imaging system based on the Bradford assay (11).

RESULTS

Vitreal levels of PEDF were determined by immunoblot (Fig. 1). We detected a protein band of 50 kDa corresponding to the predicted molecular mass of PEDF. The band disappeared or was diminished after preincubation of the antibody with recombinant PEDF, thereby demonstrating specificity of the reaction. Recombinant PEDF occurred as a single band on a SDS-polyacrylamide gel as investigated by Ponceau S staining.
FIG. 2. Levels of vitreal PEDF in patients with proliferating eye disease. PEDF levels in intraocular samples from numerous patients were determined by Western blot analysis and compared with a standard concentration of purified human recombinant PEDF. The influence of intraocular activity was investigated by comparing levels of intraocular PEDF in patients with different degrees of neovascular activity; control subjects without angiogenesis, patients with quiescent PDR, patients with active PDR, and nondiabetic patients with extensive retinal neovascularization caused by central-vein occlusion (Rubeosis).

Photocoagulation replenishes intraocular levels of PEDF. Previous photocoagulation was associated with reduced neovascular activity (Fig. 3). Although 70% of the patients without prior photocoagulation (PDR — PRP) suffered from active angiogenesis, only 30% of the patients with PDR + PRP had active neovascularization. Patients with PDR + PRP had higher concentrations of PEDF \( p = 0.01 \) compared with patients with PDR — PRP \( p = 0.007 \). However, PEDF concentrations of patients with previous photocoagulation were still clearly below levels of control patients \( p = 0.007 \).

PEDF levels are associated with the localization of retinal neovascularization. Taking all patients into account, levels of PEDF correlated significantly with the localization of retinal neovascularization. Patients with NVE and NVD \( (18 \pm 0.4 \text{ nmol/l}, n = 20) \) had decreased levels compared with control patients without proliferation \( (23.7 \pm 0.7 \text{ nmol/l}, n = 19; P < 0.001) \) and patients with NVE only \( (21 \pm 1 \text{ nmol/l}, n = 18; P = 0.02) \). Patients with NVE or NVD only \( (19 \pm 1 \text{ nmol/l}, n = 7) \) had lower levels than control patients \( (P = 0.003 \text{ and } P = 0.002, \text{ respectively}) \). We found no correlation between vitreal levels of PEDF and sex, duration of diabetes, Hba1c, or age of the patients.

PEDF-specific immunohistochemistry of human retinas with different stages of diabetic retinopathy. To obtain data about spatial and temporal changes of PEDF expression in the course of diabetic retinopathy, 25 specimens of human retina were examined by immunohistochemistry. Our results revealed an interstitial accumulation of PEDF in the eyes of control subjects (Fig. 5A), patients with diabetes without ocular abnormalities (Fig. 5F), and patients with nonproliferative diabetic retinopathy (NPDR) (Fig. 5C), thereby confirming the murine staining pattern previously described (7). We qualitatively assessed the staining for each section (as described in research design and methods), and our results show that intraretinal intensity of staining was nearly abolished in patients with PDR (mean 0.4 [range 0–1]) (Fig. 5D) compared with control subjects (2.2 [1–3]), patients with diabetes without ocular disease (1.6 [1–2]), and patients with NPDR (1.2 [1–2]), despite unchanged intensity of unspecific staining of the fibrous tissue. Patients with previous scatter photocoagulation resulting in quiescent PDR had weak intraretinal immunochromatography that was, on average, slightly more intense (1.0 [0–2]) (Fig. 5E) than that of patients with active PDR.

Taken together, our results demonstrate a significant intraocular loss of the angiogenesis inhibitor PEDF in patients with neovascularizing eye disease such as PDR. Intraocular levels of PEDF strongly correlate with the degree of retinal neovascularization. In addition, we demonstrate that retinal scatter photocoagulation, the treatment of choice for patients with diabetic retinopathy, replenishes concentrations of PEDF in the eye. Changes of vitreal levels are confirmed by immunohistochemistry, which reveals an interstitial staining pattern as expected for a secreted protein.

DISCUSSION

The switch to an angiogenic phenotype of proliferating tissues requires both upregulation of angiogenic stimulators and downregulation of angiogenesis inhibitors. An elevated expression of angiogenic growth factors such as vascular endothelial growth factor (VEGF) in patients with retinal neovascularization has been previously demon-
FIG. 4. Concentrations of PEDF in human vitreous depending on localization of neovascularization. PEDF levels were determined as described in Fig. 2. Levels of PEDF correlate with the localization of retinal neovascularization. We compared patients without retinal angiogenesis (control subjects), patients with NVD, those with NVE, and those with both NVE and NVD.

stratified (4). Additionally, decreased expression of VEGF was observed in patients with reduced neovascular activity after panretinal photocoagulation (2). The question targeted by this study was whether a loss of angiostatic growth factors such as PEDF is critical in the development of retinal neovascularization in vivo in humans. We found PEDF concentrations in ocular fluid to be lower in patients with active neovascularization than in control subjects without retinal angiogenesis. The vitreal data are confirmed by the results of immunohistochemistry showing almost no staining in patients with active proliferation compared with a strong intraretinal staining in control patients. These results demonstrate regulation of the major intraocular angiogenesis inhibitor PEDF in vivo depending on the stage of retinal ischemia. The data support the concept that induction of angiogenesis in the human eye requires not only elevation of angiogenic growth factors such as VEGF (4) but also a decrease in angiogenesis inhibitors such as PEDF (1,7). PEDF has been proposed to be an age-dependent regulated protein (10). However, our data do not support this concept, although the number of control patients in our study may be too small to definitively answer this question.

We found that intraocular PEDF levels were reduced in nondiabetic patients with severe retinal ischemia caused by central-vein occlusion. Therefore, hypoxia rather than hyperglycemia promotes intraocular reduction of PEDF in humans. Our immunohistochemical findings show a small reduction in staining intensity in diabetic patients without retinal alterations and in diabetic patients with nonproliferative abnormalities (such as microaneurysms) compared with control subjects. These results suggest that glycemic control might also influence the expression of PEDF in the eye. Because of technical reasons in regard to quantification of immunohistochemistry in general, we cannot fully exclude small differences in the expression of PEDF in NPDR compared with control patients. Even such small differences might be relevant in the early stages of diabetic retinopathy, as suggested by data showing PEDF-dependent functional changes of retinal vessels (L.P. Aiello, Boston, MA; personal communication).

An important observation of this study was that patients with quiescent retinal neovascularization who mostly had retinal photocoagulation before intraretinal surgery had higher levels of PEDF compared with patients with active neovascularization without previous photocoagulation. Retinal photocoagulation induces regression of retinal neovascularization and has been shown to be associated with a reduction in the incidence of severe visual loss and retinal neovascularization (12). In our study group, patients with previous photocoagulation had reduced neovascular activity compared with patients without prior photocoagulation, suggesting that the positive effects of retinal photocoagulation are mediated at least in part by the reestablishment of near-normal PEDF levels. Presumably, a reduction in retinal ischemia after photocoagula-

FIG. 5. PEDF protein expression in retinal samples from patients with different stages of diabetic retinopathy. Human retinas were examined by immunohistochemistry for PEDF expression. Staining patterns for representative sections are shown for control subjects (A), patients with diabetes without ocular abnormalities (B), and patients with NPDR (C) compared with patients with PDR (D) and patients with quiescent PDR after retinal photocoagulation (F). Specificity of reaction was demonstrated by the absence of staining after incubating sections with the primary antibody that was preabsorbed with the recombinant antigen (F).
tion increases expression of angiogenesis inhibitors such as PEDF, thereby further suppressing neovascular activity. Indeed, PEDF expression was initially induced by hyperoxia in neonatal mice (7). However, the patients in our study still exhibited intraocular proliferative activity requiring intraocular surgery. PEDF concentrations of patients after retinal scatter photocoagulation remained below those of control patients, thereby possibly explaining further existing proliferative activity in the subjects investigated.

A receptor for PEDF has not yet been identified, although radio-ligand binding studies in retinoblastoma cells and cerebellar granule neurons suggest a PEDF-specific receptor (13). Until now there has been no information about binding properties of putative receptors on vascular cells, putative binding proteins, or specific biological activities on different vascular cell types. Intraocular levels in mice are as high as 90 nmol/l. Despite these comparably high levels, systemically administered PEDF was able to completely inhibit aberrant retinal angiogenesis in a model of ischemia-induced proliferative retinopathy (8). This clearly indicates that increasing PEDF levels in the murine eye by systemic substitution is therapeutically effective. Our results with a loss of PEDF in humans strongly suggest that a similar PEDF-based treatment might be a promising therapeutic approach in patients with neovascularizing eye disease. Clearly, further investigations are needed to identify the exact mechanisms of PEDF release, PEDF-induced biological effects, and possible PEDF binding to putative binding proteins in the vitreous, such as that described for IGFs.

In conclusion, PEDF meets the criteria hypothesized for an ischemia-suppressed antiangiogenic factor (1). This principle, with obvious therapeutic impact, has been confirmed in animal studies (8). Here we suggest that the loss of a major angiogenesis inhibitor in the eye, PEDF, has a central role in vivo in humans in mediating the angiogenic response of retinal ischemia, such as that seen in PDR and other ischemic retinal disorders. In addition to the previously observed changes in angiogenic growth factors such as VEGF, our data support the hypothesis that an imbalance in the angiogenic ratio between angiogenic and antiangiogenic growth factors contributes significantly to the development of retinal neovascularization. Our data might potentially induce further investigations into the effectiveness of PEDF substitution in humans. Further characterization of ischemia-regulated PEDF expression and its biological effects should offer hopeful new therapeutic approaches to prevent blindness in patients with neovascularizing eye disease.

ACKNOWLEDGMENTS

This work was supported by the German Diabetes Association (to J.S.), the Eli Lilly International Foundation (to J.S. and A.F.H.P.), and the Wellcome Trust (to M.B.). The PEDF-plasmid was kindly provided by N. Bouck.

REFERENCES


EXTENDED REPORT

Vascular endothelial growth factor C promotes survival of retinal vascular endothelial cells via vascular endothelial growth factor receptor-2

Bojun Zhao, Gill Smith, Jun Cai, Aihua Ma, Mike Boulton

Aim: To determine vascular endothelial growth factor C (VEGF-C) expression in retinal endothelial cells, its antiapoptotic potential and its putative role in diabetic retinopathy.

Method: Cultured retinal endothelial cells and pericytes were exposed to tumour necrosis factor (TNFα) and VEGF-C expression determined by reverse transcriptase-polymerase chain reaction. Secreted VEGF-C protein levels in conditioned media from endothelial cells were examined by western blotting analysis. The ability of VEGF-C to prevent apoptosis induced by TNFα or hyperglycaemia in endothelial cells was assessed by flow cytometry. The expression of VEGF-C in diabetic retinopathy was studied by immunohistochemistry of retinal tissue.

Results: VEGF-C was expressed by both vascular endothelial cells and pericytes. TNFα up regulated both VEGF-C and vascular endothelial growth factor receptor-2 (VEGFR-2) expression in endothelial cells in a dose-dependent manner, but had no effect on VEGFR-3. Flow cytometry results showed that VEGF-C prevented endothelial cell apoptosis induced by TNFα and hyperglycaemia and that the antiapoptotic effect was mainly via VEGFR-2. In pericytes, the expression of VEGF-C mRNA remained stable on exogenous TNFα treatment. VEGF-C immunostaining was increased in retinal vessels in specimens with diabetes compared with retinal specimens from controls without diabetes.

Conclusion: In retinal endothelial cells, TNFα stimulates the expression of VEGF-C, which in turn protects endothelial cells from apoptosis induced by TNFα or hyperglycaemia via VEGFR-2 and thus helps sustain retinal neovascularisation.

Vascular endothelial growth factor A (VEGF-A) plays a key part in diabetic retinopathy by increasing retinal vascular permeability and inducing neovascularisation. However, the inhibition of VEGF-A only partially decreases neovascularisation and vessel hyperpermeability, suggesting that other VEGF family members may also be involved in this process. VEGF-C is a member of the VEGF family that displays a high degree of homology with VEGF-A. The VEGF-C precursor binds only vascular endothelial growth factor receptor (VEGFR)-3, whereas the fully processed VEGF-C ligand can bind and activate both VEGFR-2 and VEGFR-3. VEGF-C stimulates proliferation and migration of blood vascular endothelial cells and promotes release of nitric oxide and plasminogen activator from endothelial cells. In animal models, VEGF-C induces angiogenesis and increases vascular permeability. Furthermore, high expression of the VEGF-C protein and gene has been found in different vascularised tumour tissues. The activation of both VEGFR-2 and VEGFR-3 has been implicated in angiogenesis, and VEGFR-3 is present in different vascular beds including the retinal vasculature. The pathogenesis of diabetic retinopathy may be correlated with chronic subclinical inflammation, and anti-inflammatory drugs have been shown to prevent early diabetic retinopathy via tumour necrosis factor (TNFα) suppression. TNFα has been found in human retinas with proliferative eye diseases and in animal models of retinal neovascularisation. Furthermore, hyperglycaemia also plays an important part in the onset and progression of diabetic retinopathy by inducing apoptosis of vascular cells, advanced glycation end product deposition and up regulation of angiogenic factors.

This paper reports that VEGF-C can promote survival of retinal endothelial cells and that this can be regulated by both TNFα and hyperglycaemia.

MATERIALS AND METHODS

Reagents

Recombinant TNFα, an anti-VEGFR-2 neutralising antibody, recombinant VEGF-C wild type (which binds both VEGFR-2 and VEGFR-3) and VEGF-C (Cys156Ser; a selective agonist of VEGFR-3) were obtained from R&D Systems Europe (Abingdon, UK). Anti-VEGF-C antibody was from Santa Cruz (UK). For immunohistochemistry, an affinity-purified goat polyclonal antibody raised against the carboxy terminus of the VEGF-C precursor of human origin (c-20) was obtained from Autogen Bioclear (Calne, Wiltshire, UK). TRizol was from Invitrogen (Glasgow, UK), and polymerase chain reaction (PCR) Reddy Mix and Master Mix Kit were purchased from Abgene (UK). Vybrant apoptosis Assay Kit was from Molecular Probes (UK). All other materials were from Sigma unless otherwise stated.

Cell culture

Primary cultures of bovine microvascular retinal endothelial cells (MECs) and pericytes were isolated as described previously. Endothelial cells were maintained in an endothelial environment.
cell basal medium with growth supplement (TCS Works, Buckingham, UK). Cells were characterised by their cobblestone-appearance and expression of factor VII antigen. Pericytes were cultured in Eagle's minimal essential medium (GibcoBRL, Paisley, UK) containing 10% fetal calf serum. Pericytes were identified and distinguished from endothelial cells by their size, irregular morphology and negative staining for factor VIII. Both cell types were used between passages 1 and 3 for all experiments.

To ensure cross species recognition of VEGFRs, primary cultures from human donor eyes were obtained from the Bristol Eye Bank, Bristol, UK, and used in accordance with the tenets of the Declaration of Helsinki. The cultures were isolated and maintained as described above and used for the apoptosis studies within five passages.

TNFα treatment
For gene and protein expression studies, cells were treated with different concentrations of TNFα for up to 6 h. For time-dependent studies of TNFα treatment, cells were incubated with 10 ng/ml TNFα at different time points.

Reverse transcriptase-polymerase chain reaction
To investigate gene expression of VEGF-C and its receptors, total RNA was isolated from endothelial cells and pericytes exposed to different experimental conditions, using the isolation kit TRIzol, and then analysed by reverse transcription-polymerase chain reaction (RT-PCR) using the First Strand Synthesis Kit and PCR ReddyMix according to the manufacturer's protocol. Equal quantities of total RNA were used from different samples. The primers for VEGF-C were according to the sequences of bovine VEGF-C from GenBank. The oligonucleotide primers for amplification of VEGF-C cDNA were 5'-GAA CAA GGG TTA TGC AGG AA-3' and 5'-CCA CAT TCT TAG ACG GAC ACA C-3'. The resultant PCR product was 348 bp long. The primers for VEGFR-2 were from Berisha et al.11 11 and VEGFR-3 was from Pepper et al.11 11 The sequences were 5'-TGT TCC AGT ATG ATT CCA CCC-3' and 5'-TCC ACC CTG TTT TGC TA-3'. The cDNA was amplified using the PCR Master Mix, each cycle consisting of 20 s at 94°C, 30 s at 55°C and 30 s at 72°C. All the samples were amplified in a linear amplification range established using a serial cDNA dilution and varying the number of cycles. PCR products were electrophoresed on a 1.2% agarose gel containing ethidium bromide and visualised under ultraviolet light. The relative intensities of the bands were quantified by densiometric analysis.

Immunoprecipitation and western blotting
To investigate gene expression of VEGF-C and its receptors, the conditioned media was collected and subjected to immunoprecipitation and western blotting after 24-h of treatment. The media from parallel samples was collected and subjected to the apoptosis assay according to the manufacturer's instructions. The samples were analysed using a fluorescent activated cell sorting 450 Flow Cytometer (Becton Dickinson, Oxford, UK). Viable cells were double-negative stained, early apoptotic cells stained positive for annexin V and negative for propidium iodide, whereas, late apoptotic/necrotic cells were double-positive stained for annexin V and propidium iodide. To define the role of VEGFR-2 in the anti-apoptotic effect of VEGF-C, 60 ng/ml anti-VEGFR-2 antibody was added to the culture medium for 1 h before incubation with 100 ng/ml TNFαs and 200 ng/ml VEGF-C, or 30 mM glucose and 200 ng/ml VEGF-C. Anti-VEGFR-2 antibody alone acted as a control. To observe whether VEGFR-3 had an anti-apoptotic function, 200 ng/ml VEGF-C (Cys156Ser; a selective agonist of VEGFR-3) was administrated together with 100 ng/ml TNFαs for 48 h.

To block the basal secretion of VEGF-C, cells were transfected with either VEGF-C small interfering RNA (siRNA) or scrambled siRNA for 72 h and then incubated with 100 ng/ml TNFαs for 48 h. siRNA duplexes were designed and synthesised by Dharmacon Research (Lafayette, Colorado, USA) to target the bovine sequence of VEGF-C 5'-ACA GAG ATC TTA AGA AGT A-3'. The premade siRNA (scramble II; Dharmacon) was used as a negative control. Cells were transfected with siRNA duplexes using DharmaFECT 1 (Dharmacon) at a final RNA concentration of 100 nmol/l according to the manufacturer's protocol. To determine the efficiency of transfection, the medium from parallel samples was collected and subjected to immunoprecipitation and western blotting after 24-h of incubation.

Immunohistochemistry
A total of 47 eyes enucleated and fixed in 10% neutral-buffered formalin within 10 h after death were obtained from the National Disease Research Interchange, Philadelphia, USA. All procedures were performed according to the Declaration of Helsinki. Eyes were categorised by an ophthalmologist based on fundus appearance as normal (no known ophthalmic disease, no history of diabetes, no abnormalities on biomicroscopy), diabetic with no overt retinopathy, diabetic with intraretinal changes but no evidence of proliferative diabetic retinopathy (PDR), diabetic with periretinal PDR and diabetic with scatter laser photocoagulation but no evidence of residual PDR.14 14

Immunohistochemistry was performed on 5 μm sections as described previously.15 Sections were incubated overnight at 4°C with a polyclonal VEGF-C antibody (2 μg/ml). The negative control was the substitution of the primary antibody with an inappropriate rabbit IgG. After washing, sections were incubated for 30 min with biotinylated rabbit anti-goat IgG, then for a further 30 min with alkaline phosphatase reaction mixture (Dako) and incubated with Fast Red TR (Sigma, UK). Sections were counterstained with Mayer's haematoxylin. The degree and pattern of immunostaining was assessed by two blinded observers. The intensity of staining was graded qualitatively as background, weak, moderate or intense (corresponding to the highest level of immunoreactivity).
Figure 1  The regulation of vascular endothelial growth factor-C (VEGF-C) mRNA expression by tumour necrosis factor (TNF)α in microvascular endothelial cells and pericytes. (A) Dose-response of mRNA induction of VEGF-C by TNFα in microvascular retinal endothelial cells (MECs). MECs were stimulated with the indicated concentrations of TNFα for 6 h. (B) Time dependence of VEGF-C mRNA induction by TNFα. MECs were stimulated with TNFα (10 ng/ml) for 0-24 h. (C) Expression of VEGF-C mRNA in bovine retinal pericytes after exposure to different concentrations of TNFα for 6 h. The isolated total RNA from different treatments was subjected to reverse transcriptase-polymerase chain reaction and polymerase chain reaction products were analysed by agarose gel electrophoresis. Band intensities were quantified by laser densitometry. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the internal control. Representative results from three separate experiments are shown. Vertical bars represent mean (standard error of the mean); **p<0.01, indicating significant difference between treatment and controls.

Statistical analysis
The results represent the mean of at least three separate experiments. Statistical analysis was carried out using an unpaired Student’s t test. Significance was defined as p<0.05. All numerical results are expressed as mean (standard error of the mean).

RESULTS
Regulation of VEGF C mRNA expression by TNFα in microvascular endothelial cells and pericytes
RT-PCR analysis showed that VEGF-C mRNA was expressed in MECs. TNFα stimulated the expression of VEGF-C mRNA in a dose-dependent manner, with a maximal 4.6 (0.5)-fold increase with 10 ng/ml TNFα (fig 1A). Stimulation of cells with 10 ng/ml TNFα increased VEGF-C mRNA expression in a time-dependent manner with a maximum at 6 h. Beyond 6 h, VEGF-C expression decreased, but even after 24 h stimulation, the expression of VEGF-C mRNA was still higher than that with no stimulation (fig 1B). Pericytes expressed VEGF-C mRNA, but TNFα had no regulatory effect on this expression (fig 1C).

Increased expression of VEGFR-2, but not VEGFR-3 mRNA in MECs challenged with TNFα
RT-PCR results showed that MECs expressed both VEGFR-2 and VEGFR-3 mRNA (fig 2). TNFα induced an increase of VEGFR-2 mRNA in a dose-dependent manner. The levels of VEGFR-2 mRNA began to increase at 1 ng/ml TNFα and reached a maximum level at 50 ng/ml TNFα (fig 2A). By contrast, the expression of VEGFR-3 was not modified by exposure to TNFα (fig 2B). The expression of VEGFR-2 and VEGFR-3 mRNA was not detectable in pericytes (data not shown).

TNFα increases VEGF C protein synthesis and secretion in MECs
Western blotting showed that the secreted peptide was present at high amounts, with a maximum 4.1 (0.4)-fold increase in the medium under TNFα conditions compared with the medium of control cultures (fig 3A). Treatment with cycloheximide considerably reduced the amount of VEGF-C in the conditioned medium. This result indicates that increased amounts of VEGF-C released by MECs in response to TNFα treatment are due to
VEGF-C promotes endothelial cell survival

**Figure 2** Tumour necrosis factor (TNF-α) stimulates the expression of vascular endothelial growth factor receptor (VEGFR)-2 mRNA, but not VEGFR-3 in microvascular retinal endothelial cells (MECs). Total RNA was isolated 6 h after stimulation by different concentrations of TNF-α. Reverse transcriptase-polymerase chain reaction was performed and polymerase chain reaction products were analysed by agarose gel electrophoresis. The signal intensity was determined by densitometry, and the amount of VEGFR-2/VEGFR-3 was normalised for the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) present. Representative results from at least three separate experiments are shown. Vertical bars represent mean (standard error of the mean); *p<0.05 and **p<0.01, indicating significant difference between treatment and controls. (A) Expression of VEGFR-2 mRNA; (B) expression of VEGFR-3 mRNA.

Increased protein synthesis rather than an increased release of VEGF-C from cell storage (fig 3B)

**VEGF C prevents TNFα and hyperglycaemia-induced apoptosis in MECs and this effect occurs mainly via VEGFR-2**

Flow cytometry showed that TNFα induced apoptosis/necrosis and that this was markedly inhibited by VEGF-C. The cell population at the late stage of apoptosis/necrosis reduced from 56.5% (1.38%) to 28.1% (0.7%) when VEGF-C was present (fig 4A–C). The apoptotic/necrotic population increased to 82.7% (2.8%) after exposure to TNFα in cells treated with VEGF-C siRNA compared with 57.9% (1.72%) in cells treated with scrambled siRNA (fig 4D,E). The efficiency of knockdown of VEGF-C expression in culture medium with RNAi was confirmed using immunoprecipitation and western blotting (fig 4L).

To identify which of the two VEGF-C receptors was responsible for the anti-apoptotic effect of VEGF-C, VEGFR-2 was blocked by neutralising antibody or cells were treated with a VEGFR-3 agonist. After neutralising VEGFR-2, the protective effect of VEGF-C on TNFα-induced apoptosis was considerably reduced. The population of apoptotic/necrotic cells was increased from 28.1% (0.7%) to 53.2% (1.41%) (fig 4C,F). VEGFR-2-neutralising antibody alone had no effect on promoting apoptosis/necrosis (fig 4G). After addition of VEGF-C (Cys156Ser), there was no protective effect on TNFα-induced apoptosis. The population of apoptotic/necrotic cells was 55.7% (2.1%), and showed no statistically significant change from cells treated with TNFα alone (fig 4B,H). After addition of VEGF-C, the percentage of apoptosis/necrosis induced by high glucose was reduced from 47.1% (1.6%) to 23.6% (1.2%) (fig 4I) and this rescue effect was abolished when VEGFR-2 was blocked by its neutralising antibody (fig 4K). Results were similar for both human and bovine MECs.

**Expression of VEGF-C in diabetic retinopathy**

Weak to moderate staining for VEGF-C was observed in the vessels of non-diabetic retinas and in retinas without overt retinopathy; staining was increased (moderate to intense) compared with non-diabetic retinas once intraretinal changes became obvious (4 of 5 eyes) and markedly increased in PDR retinas (6 of 6 eyes; table 1, fig 5). Intense staining was also

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**Figure 3** Western blotting analysis of de novo vascular endothelial growth factor-C (VEGF-C) protein synthesis. (A) Microvascular retinal endothelial cells (MECs) were treated with 1 or 10 ng/ml tumour necrosis factor (TNF)-α for 24 h. MECs were treated with 10 ng/ml TNFα and 3.6 μM cyclohexamide (CHX) for 24 h, and 10 ng/ml TNFα alone was the control. Conditioned media were immunoprecipitated using a polyclonal antibody raised to VEGF-C and the immunoprecipitates electrophoresed by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, followed by blotting on a nitrocellulose membrane. Positive bands were visualised by an enhanced chemiluminescence reaction detection system. Band intensity for VEGF-C was quantified by laser densitometry from at least three separate experiments. Vertical bars represent mean (standard error of the mean); **p<0.01, indicating significant difference between treatment and controls.

Increased protein synthesis rather than an increased release of VEGF-C from cell storage (fig 3B)
Figure 4  (A–K) Vascular endothelial growth factor (VEGF)-C prevents tumour necrosis factor (TNFα)-induced apoptosis via vascular endothelial growth factor receptor (VEGFR)-2. Microvascular retinal endothelial cell (MECs) were given different treatments for 48 h. The cells were then stained with annexin V-fluorescein isothiocyanate conjugate (FITC) containing propidium iodide (PI). Data show two-parameter analysis of fluorescence intensity of annexin V and PI. Annexin V/PI-negative cells were counted as viable cells (lower left quadrant). All measurements were performed in triplicate. Representative results from at least three separate experiments are shown. (A) Control; (B) TNFα; (C) TNFα+VEGF-C; (D) TNFα+VEGF-C siRNA; (E) TNFα+scrambled siRNA; (F) TNFα+anti-VEGFR-2 antibody; (G) anti-VEGFR-2-neutralising antibody alone; (H) TNFα+VEGF-C (Cys156Ser); (I) high glucose; (J) high glucose+VEGF-C; (K) high glucose+VEGF-C+anti-VEGFR-2 antibody. Numbers in the quadrant are the percentage of FITC+/PI+ cells. (L) Knockdown of the expression of VEGF-C in culture medium from small interfering (si)RNA-treated MECs. Culture medium from either VEGF-C siRNA or scrambled siRNA-treated cells was collected after 24 h of incubation and equal quantity of proteins was subjected to immunoprecipitation and western blotting. Positive bands were quantified by laser densitometry from at least three separate experiments. Vertical bars represent mean (standard error of the mean); **p<0.01, indicating significant difference between VEGF-C siRNA treatment and scrambled siRNA.
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observed in the vessels of preretal membranes. After laser treatment, the levels of VEGF-C in the retinal vessels were reduced to weak staining in 11 of 14 eyes. In addition, increased staining was observed in the ganglion cell layer of diabetic retinas both with and without intraretinal changes as compared with the minimal staining in non-diabetic retinas. Staining was weak or absent in the choroidal vessels, the RPE, the photoreceptors, and the outer and inner retinal layers (table 1). The variability of staining within retinas of the same group did not show a correlation with donor age, the type of glycaemic control in the case of the diabetic groups or time after death.

DISCUSSION

In this study, we showed that TNFα strongly upregulates the expression of VEGF-C in MECs and that this induction is both time dependent and dose dependent. The dose–response study showed that the stimulatory effect of TNFα was produced at a concentration as low as 0.1 ng/ml, which is within the range of TNFα concentrations in vitreous fluid from patients with active PDR.10 VEGFR-2 was also upregulated by TNFα, whereas the expression of VEGFR-3 mRNA remained stable with various TNFα treatments, suggesting that in our experimental system VEGF-C may exert its angiogenic effect mainly via increasing VEGFR-2 rather than VEGFR-3. This increase in VEGFR-2 may also be important in enhancing VEGF-A-induced angiogenesis.

After blockade of VEGFR-2, the antiapoptotic effect of VEGF-C was abrogated, whereas the activation of VEGFR-3 by VEGF-C (Cys156Ser) did not attenuate TNFα-induced apoptosis showing that VEGFR-2 is the dominant receptor for VEGF-C action in MECs. Our data support the observations that VEGF-C, which was originally thought to be a potent inducer of

Figure 5 Immunolocalisation of vascular endothelial growth factor (VEGF)-C in the diabetic retina. VEGF-C staining is shown for representative retinal sections. Weak to moderate VEGF-C immunostaining was localised to non-diabetic retina (A) and diabetic retina with no obvious intraretinal vascular changes (B). By contrast, moderate to intense VEGF-C staining was observed in the diabetic retina with obvious intraretinal vascular changes but no evidence of proliferative diabetic retinopathy (PDR; C) and in the diabetic retina with PDR (D). After laser treatment, the only weak immunostaining for VEGF-C was observed (E). Immunostaining was raised in later-stage diabetic retinopathy and was generally confined to the intraretinal vessels (IRV). Immunoreactivity was abolished in the control retina processed with omission of primary antibody (F). Magnification ×200. PRM, polynomial regularisation method.

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lymphangiogenesis,\textsuperscript{7, 8} may also act as a survival factor to suppress apoptosis in vascular endothelial cells. VEGF-C has been shown to be important in vascular angiogenesis in other vascular beds,\textsuperscript{7, 8} and the response can be robust and indistinguishable from that observed using VEGF-A.\textsuperscript{9, 10} The existence of VEGF-C in human retinas and the expression of VEGF-C in the retinal vasculature that increases in diabetic retinopathy further support a role for VEGF-C in diabetic retinopathy. Interestingly, laser photocoagulation, a proved treatment for reversing neovascularisation in PDR, resulted in a marked reduction in VEGF-C protein expression.

Our data showed that mRNA coding for VEGF-C was present not only in endothelial cells but also in pericytes. However, the regulatory effects differ between the two principal types of microvascular cells. The VEGF-C gene remained constitutively expressed in pericytes on adding various concentrations of TNFα, but VEGF-C is unlikely to signal in an autocrine fashion as only VEGF-1 is expressed in pericytes\textsuperscript{11, 12}; TNFα is a macrophage-secreted monocyte-derived pluripotent mediator. Whether TNFα plays a part in angiogenesis may be highly dependent on its concentration.\textsuperscript{13} TNFα has been shown to be a powerful activator of angiogenesis in vivo in several animal models when used at appropriate doses.\textsuperscript{13, 14} Previous studies show that high glucose or advanced glycation end products induce the expression of proinflammatory cytokines, including TNFα from monocytes and macrophages,\textsuperscript{13, 16} and TNFα may have an important role in mediating angiogenesis in diabetic retinopathy.\textsuperscript{6, 17} The angiogenic effect of TNFα may be due to the generation of secondary mediators.\textsuperscript{6, 17}

In conclusion, increased expression of VEGF-C protein in the retinal vasculature of diabetic retinopathy suggests that VEGF-C may have an important role in its pathogenesis.

**Table 1** Mean (SD) intensity of vascular endothelial growth factor-C in the retina and choroids

<table>
<thead>
<tr>
<th>Choroid</th>
<th>RPE</th>
<th>Photoreceptors</th>
<th>Outer nuclear layer</th>
<th>Inner nuclear layer</th>
<th>Ganglion cell layer</th>
<th>Retinal vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-diabetic (n=14)</td>
<td>0.6 (0.8)</td>
<td>0.1 (0.4)</td>
<td>0.4 (0.8)</td>
<td>0 (0)</td>
<td>0.3 (0.4)</td>
<td>1.1 (1.2)</td>
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<td>No prior retinopathy (n=12)</td>
<td>0.8 (0.6)</td>
<td>0.1 (0.3)</td>
<td>0.3 (0.7)</td>
<td>0 (0)</td>
<td>0.1 (0.3)</td>
<td>1.2 (1.0)</td>
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<tr>
<td>Intraretinal changes (n=5)</td>
<td>0.2 (0.5)</td>
<td>0.0 (0.0)</td>
<td>0.1 (0.5)</td>
<td>0.2 (0.5)</td>
<td>0.1 (0.1)</td>
<td>1.2 (1.1)</td>
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<tr>
<td>PDR (n=6)</td>
<td>0.8 (0.8)</td>
<td>0.3 (0.5)</td>
<td>0.3 (0.5)</td>
<td>0.2 (0.4)</td>
<td>0.3 (0.3)</td>
<td>2.5 (0.3)</td>
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<tr>
<td>Laser, no residual PDR (n=14)</td>
<td>0.6 (0.6)</td>
<td>0 (0)</td>
<td>0.4 (0.6)</td>
<td>0.4 (0.9)</td>
<td>0.3 (0.5)</td>
<td>0.3 (0.2)</td>
</tr>
</tbody>
</table>

PDR, proliferative diabetic retinopathy; RPE, retinal pigment epithelium.
VEGF-C promotes endothelial cell survival


