THE ROLE OF GROWTH FACTORS IN PROLIFERATIVE DIABETIC RETINOPATHY

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Background retinopathy is an almost inevitable consequence of diabetes mellitus, but proliferative diabetic retinopathy (PDR) occurs only in 60% of diabetic patients even after 40 years of diabetes.[1] This variation in susceptibility to the different stages of diabetic retinopathy has been attributed to a requirement for a second risk factor for the development of PDR, in addition to prolonged exposure to high circulating levels of glucose. Several such co-factors have been described including elevated diastolic blood pressure, fibrinogen, low density lipoprotein, cholesterol and triglycerides, but recent epidemiological analysis has shown that the common denominator in these patients was minimal nephropathy.[2]

Current perceptions of the pathogenesis of diabetic retinopathy emphasise the role of local microvascular damage in the initiation of the disease, particularly through glucose toxicity to the endothelial cell.[3] Clearly many of the metabolic and tissue effects such as endothelial cell damage, capillary closure and retinal ischaemia in PDR can be attributed directly to initial glucose-induced microvascular cell damage. Local production of an 'angiogenic factor' by ischaemic retina, initially proposed by Michaelson,[4] is also currently considered to be the major mechanism in the development of new blood vessels in PDR, but clearly systemic factors play a part if nephropathy is truly a second risk factor for PDR. The following brief review addresses some of these issues and outlines a possible mechanism which takes into account both local and systemic factors in the development of PDR.

PATHOLOGY OF PROLIFERATIVE DIABETIC RETINOPATHY

PDR is a term which describes a range of clinical presentations of advanced diabetic retinopathy including active neovascularisation either at the optic disc or elsewhere in the retinal vasculature, regressing or involuted 'new

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vessels', flat new vessels before they have reached the stage of forward displacement into the vitreous cavity, and mature new vessels often within a field of preretinal gliosis. Several cell types may therefore be involved in 'proliferation' and cause damage including glial cells, retinal pigment epithelial cells, fibroblasts and inflammatory cells, but pathogenetically the endothelial cells of the prevenular capillories are probably the first cells to escape growth control (see later).

Proliferation of endothelial cells occurs at an early stage in diabetic retinopathy most frequently as clusters of cells which are found in the more common type of microaneurysm (Fig. 1). When solitary microaneurysms occur, they usually do so in very small regions of retinal ischaemia as revealed by fluorescein angiography (Fig. 2); as further damage develops, groups of microaneurysms surround larger patches of capillary closure and ischaemic retina (Fig. 2). Such miroaneurysms represent 'arrested' neovascular responses in comparatively healthy endothelial cells at the edge of discrete patches of retinal damage where both retinal endothelial cell and neural cell death has occurred. Foci of frank neovascularisation (angiogenesis) occur much less frequently in comparison with the numbers of microaneurysms, and it has been suggested that a certain area of retina must be involved in the ischaemic process before neovascularisation becomes ophthalmoscopically detectable. Clinically, a pre-neovascular stage is recognised as 'intraretinal microvascular abnormality' (IRMA).

Once frank neovascularisation has occurred, it usually progresses through several stages: initially 'flat' new vessels grow on the surface of the retina in the potential space between the vitreous gel and the retina (subhyaloid space); firm adhesion of the forward-migrating endothelial cells to the vitreous gel induces contraction of its collagenous matrix such that it detaches from the retinal surface (posterior vitreous detachment) (Fig. 3a); this causes forward traction of the attached immature retinal vessels which frequently leads to subhyaloid haemor-

Fig. 1. *Trypsin digest preparation of human diabetic retina showing acellular 'vascular' cords and two microaneurysms. Type A (long arrow) is the more common type and shows multiple endothelial cell nuclei representing a 'proliferative' response; Type B (short arrow) shows a blood-filled saccular outgrowing of the vessel wall.*

rhage. Histologically, forward retinal vessels are often only partially developed with ill-defined basement membrane structure and considerable encapsulation by vitreous collagen fibres (Fig. 3b). In addition there is a marked macrophage response around forward-growing retinal vessels (Fig. 3c) which is probably in part secondary to the haemorrhage but which may be important for the further growth of the vessels (see below). Recurrent subhyaloid haemorrhages occur as posterior vitreous detachment progresses and glial cells proliferate on the posterior hyaloid surface (Fig. 3c, d) and as sheets of cells across areas where vitreoretinal attachments become strong (e.g. along the vessel arcades). This causes considerable tangential/oblique traction on the retinal surface leading to retinal folds, traction retinal detachment, retinal hole formation and rhegmatogenous retinal detachment, at which point retinal pigment epithelial cells may participate in the process and add features of proliferative vitreoretinopathy (PVR) to the final histology.[5]
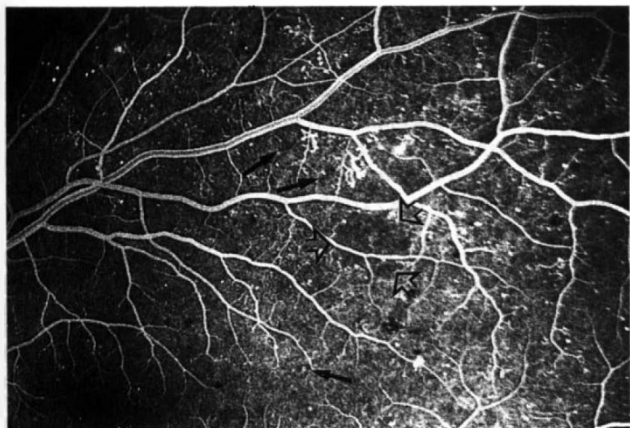

Fig. 2. *Venous phase fluorescein angiogram of diabetic fundus. There are single microaneurysms in small discrete areas of retinal ischaemia (arrows) and multiple microaneurysms surrounding a large area of retinal ischaemia (open arrowheads).*

CELLULAR EVENTS IN NEOVASCULARISATION

Angiogenesis is central to many physiological and pathological processes such as wound healing, oogenesis, embryogenesis and tumour formation. The process of angiogenesis progresses through a coordinated series of discrete steps including the following: endothelial cell activation and shape change, pericellular protease secretion and matrix degradation, endothelial cell migration, proliferation and invasion of the extracellular matrix by cords of advancing endothelial cells, and finally differentiation of the cellular cords into lumen-containing vessels which mature into capillary loops and arcades with laying down of basement membrane.[6,7] Angiogenesis requires two signals for induction and these are markedly influenced by the local microenvironment around the cell: (1) changes in the extracellular matrix which permit adhesion and migration of the cell and which may also have direct effects on the proliferative response of the cell; (2) activation and growth-promoting cytokines which initiate intracellular signals for proliferation and other responses (angiogenic factors).

'Angiogenic factors' are generally described in terms of their proliferation-inducing capacity for endothelial cells, but clearly this is too restricted since many factors modulate one or more of the above activities of endothelial cells, and depending on the conditions the same factor may have a pro- or anti-angiogenic effect as occurs with tumour necrosis factor α (see below). The same factor may also have pro- or anti-angiogenic effects on different stages in the angiogenic process, as for instance with transforming growth factor β which is inhibitory for endothelial cell proliferation but promotes vessel maturation. It is therefore necessary to define the particular 'angiogenic' effect for each factor or cytokine. Tables I and II list some of the known factors which modulate the angiogenic response and the following sections describe some of these factors in more detail.

MATRIX COMPONENTS AFFECTING ANGIOGENESIS

Extracellular matrix cues dominate over soluble cues in growth and differentiation responses of endothelial and other anchorage-dependent cells. In some respects, the extracellular matrix controls the responsiveness of endothelial cells to soluble factors.[8] Matrix components bind to specific cell surface receptors which may initiate second messenger signals, e.g. via tyrosine kinase-mediated protein phosphorylation, thereby inducing specific cell responses. Alternatively, matrix factors may interact directly with cytoskeletal elements within the cell such as actin or talin, which then cause alterations in cell shape and state of activation. A balance exists between forces generated by contractile microfilaments within the cell and adhesive interactions with the extracellular matrix. If this balance is tilted in one direction, the cell may be induced to spread and adhere extensively to the substrate thereby becoming non-motile. A change in the other direc-
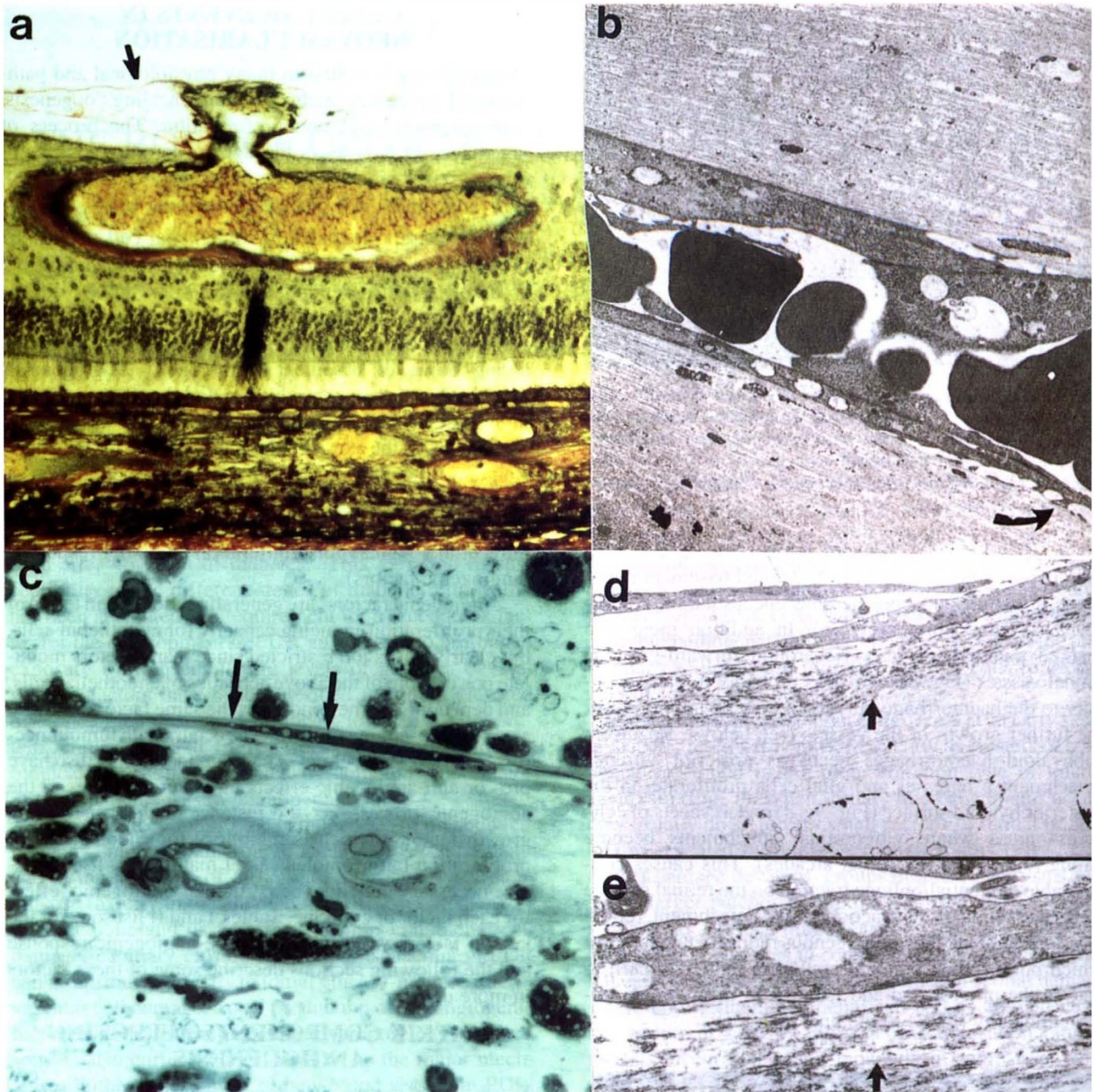
Fig. 3. *Pathology of diabetic retinal neovascularisation. (a) Light microscopic view of 'forward' new vessel in subhyaloid space with firm attachment to vitreous collagen fibres (arrow). (b) Electron micrograph of poorly developed 'new' vessel surrounded by condensed vitreous collagen and showing atypical 'foot-processes' adhering to collagenous matrix (curved arrow). (c) Intravitreal 'mature' forward 'new' vessels, showing thickening of basement membrane and associated macrophage response. Spindle-shaped glial cells line the detached posterior vitreous face (arrows). (d) Electron micrograph of glial cells lining the detached posterior vitreous face, and associated with electron-dense material within the collagen gel (arrow). (e) Higher magnification of (d) to show flocculent electron-dense material.*

tion might cause the cell to round up and migrate, e.g. up a concentration gradient of chemotactic factor.

Quiescent endothelial cells lining a vessel wall are normally non-motile, and adhere to a basement membrane composed of several matrix components. Adhesion of the cell to matrix proteins is mediated by short peptide sequences in the native protein interacting with specific receptors in the cell membrane. A large class of cell adhesion receptors known as the integrins occurs on all cells,

with different receptors being expressed on different cell types. Endothelial cell integrin expression is upregulated when these cells are activated as in inflammation and this determines the nature and level of interaction between inflammatory leucocytes and the endothelium.

Degradation of the basement membrane in the initial stages of angiogenesis exposes activated endothelial cells to proteins not normally present in the basement membrane and to peptide products of membrane dissolution.

Table I. Matrix factors involved in angiogenesis

Matrix factor	Endothelial cell response				
	Activation	Enzyme release/expression	Migration	Proliferation	Differentiation
Collagen I	−	−	−	(+)	+
Collagen III			+	+	
Collagen IV	−	−	(−)	−	+
HSPG	−	−	−	(+)	+
Hyaluronate			(−) (+)	(−) (+)	−
Fibronectin	+		+	+	+
Laminin			+	+	+
Thrombospondin		(+)	−	−	−
Entactin			?	?	?
PAI-1	−	−	−		
α2-macroglobulin					

+, stimulation; −, inhibition; (−) (+), possible effect; ?, unknown; blank, not tested.
HSPG, heparan sulphate proteoglycan; PAI-1, plasminogen activator 1.

Many of these proteins are permissive for cell activities associated with the various stages of angiogenesis.

Laminin

Laminin, a major component of the basement membrane, occurs as a high molecular weight (>800 kDa) highly glycosylated glycoprotein composed of three different chains (heterotrimeric) with a suggested cross-like three-dimensional structure.[9] Laminin has separate domains which bind collagen, heparin and entactin, and it has two cell binding domains, one constitutively expressed on the A chain of the native molecule and a second cryptic domain which resides at the cross-section of the molecule and is only apparent after partial proteolysis.[10]

Laminin is an ideal substrate for adhesion and growth of retinal capillary endothelial cells cultured from microvessel explants in vitro,[11] but glycation of the molecule considerably reduces cell adhesiveness (Grant, McIntosh and Forrester, unpublished observations). Several adhesion-recognition sequences have been identified, usually comprising linear sequences of 5–20 amino acids, and there is evidence that these peptides may show some degree of cell specificity.[12] Laminin binding to the cell may occur via integrin receptors ($\alpha3\beta1$, $\alpha6\beta1$, $\alpha1\beta1$) and non-integrin laminin-binding proteins (67 kDa and 35 kDa membrane proteins).[13] Unlike other adhesion proteins, laminin possesses eight different binding peptide sequences, indicating that it probably acts as a binding protein for several cells and matrix molecules at one time while simultaneously providing a compact meshwork for this purpose.

As indicated above, laminin promotes endothelial cell growth and certain laminin peptides have been shown to induce angiogenesis.[14] One peptide (YIGSR, Tyr-Isoleu-Gly-Ser-Arg) promotes differentiation/maturation in endothelial cells, while a second peptide (RGD, Arg-Gly-Asp) promotes endothelial cell adhesion.[15] This latter peptide is only available to the cell after limited proteolysis, which may therefore be a prerequisite for the initiation of angiogenesis during the stage of basement membrane

Table II. Growth factors involved in angiogenesis

Growth factor	Endothelial cell response				
	Activation	Enzyme release/expression	Migration	Proliferation	Differentiation
FGF (1–7)	+	+	+	+	+
HBGF	+	+	+	+	?
Insulin	+			+	
IGF-I	+			+	
TGFβ (1–6)	−	−	−	−	+
TGFα	−				
TNFα	+	+	+	+	−
PDGF			(+)	(+)	
PDECGF			+	+	+
ECGF	+	+		+	
IL-1	+			+	
IL-6				+	
Angiogenin	+	+		+	
Vasculotropin (VEGF, VPF)			+	+	+
Platelet factor 4				−	−

+, stimulation; −, inhibition; (+) (−), possible effect; ?, unknown; blank, not tested.
FGF, fibroblast growth factor; HBGF, heparin binding growth factor; IGF-I, insulin-like growth factor I; TGF, transforming growth factor; TNF, tumour necrosis factor; PDGF, platelet-derived growth factor; PDECGF, platelet-derived endothelial cell growth factor; ECGF, endothelial cell growth factor; IL, interleukin; VEGF, vascular endothelial cell growth factor; VPF, vascular permeability factor.

degradation. Recently, a third peptide sequence from laminin (IKVAV, Isoleu-Lys-Val-Ala-Val) has been implicated in tumour angiogenesis, possibly via activation of cell surface plasminogen activator.[14,16] These results support the notion that laminin degradation products promote angiogenesis, and it is possible that the corollary is also true, i.e. that native, undegraded laminin is anti-angiogenic for endothelial cells; otherwise there would be uncontrolled proliferation of these cells. However, it may be that other matrix molecules exert a regulatory effect over laminin (see below). Laminin, like fibronectin, not only promotes cell adhesion but also induces signal transduction, and the site of activity appears to be close to the IKVAV-containing site in the molecule.[17]

Fibronectin

Fibronectin is a 450 kDa glycoprotein consisting of two identical polypeptide chains held together by disulphide bridges.[18] It occurs in soluble form in high concentration in the plasma and is also present on the surface of many cell types. It is present in multimeric form as an insoluble component of the connective tissue matrix but it is not usually considered an integral component of the basement membrane, although it may be present in significant amounts, particularly in certain pathological conditions, including diabetes.[19]

Fibronectin has several domains which are involved variously in cell binding (two sites), heparin(an) binding (two sites), fibrin(ogen) binding (two sites) and collagen binding.[12] A number of peptide sequences have been identified which may be involved in cell binding including an RGDS (Arg-Gly-Asp-Ser) sequence which is common to many proteins including laminin,[15] collagen,[20] vitronectin,[21] fibrinogen[22] and entactin.[23] The RGD sequence is not only involved in cell attachment but may also have a role to play in migration and in second messenger signalling, where its interaction with the receptors, $\alpha5\beta1,\alpha2\beta1$ integrins, has been shown to initiate cell growth and enzyme secretion.[24] Fibronectin has other adhesion recognition sequences which utilise different integrins for cell binding (e.g. Arg-Gly-Asp-Val and $\alpha4\beta1$).[25]

In the interstitial matrix, fibronectin adopts a fibrillar morphology which is induced by alignment of fibronectin molecules using the cell-binding domain and the amino terminal; thus clustered fibrils of the molecule become co-aligned with the microfilament bundles of the cell.[26] More recent studies have shown that both the cell binding and a heparin (heparan) binding domain are required to produce a fibronectin matrix.[27] Adsorption of soluble fibronectin induces a conformational change in the molecule exposing a sulphydryl group[28] which may be involved in the formation of intercellular fibronectin links during wound contraction.[29]

Most research on the adhesive properties of fibronectin has been conducted with fibroblasts and other connective tissue cell lines. Microvascular endothelial cells also require a suitable matrix, and fibronectin has been shown to be an excellent substrate for retinal vascular endothelial

cell growth and migration.[11,30] Fibronectin appears to be haptotactic for endothelial cells and is likely to have a significant effect in PDR where capillary leakage will result in adsorption of plasma fibronectin to the retinal extracellular matrix.

Collagen

Several types of collagen are involved in different stages of the angiogenic response and these may have pro- or anti-angiogenic effects. Connective tissue matrices contain fibrillar collagens (types I, II, III and V) while basement membranes contain predominantly non-fibrillar type IV, with some contribution from types VI, VII and VIII depending on the tissue.[31] The more recently described collagens (types IX–XIII) represent fibril-associated collagens which act as molecular bridges between the fibrillar collagens.[32] In addition to forming three-dimensional arrays with other matrix molecules, such as fibronectin and proteoglycans in interstitial tissues, and laminin and heparan sulphate proteoglycan in basement membranes, collagens have specific binding sites for cells including the RGD sequence.[20] Cell binding occurs via integrin-mediated and non-integrin-mediated mechanisms.[33,34]

Collagen has profound effects on endothelial cell behaviour. Whereas type I collagen provides a good substrate for retinal endothelial cell growth, cell migration on the same substrate is poor unless the collagen is present as a three-dimensional gel.[11] Even then, the cells appear to migrate singly and to adopt an elongated, fibroblast-like morphology exerting traction on the collagen fibrils. Cell migration on type IV collagen is inhibited, an effect which supersedes the migration- and growth-promoting effects of laminin even when the laminin overlies the collagen.[11] Phenotypic modulation of endothelial cells from the proliferative 'cobblestone' appearance to the 'sprouting' morphology and later the 'tube-like' capillary structures *in vitro*, is accompanied by a change in endothelial cell synthesis of collagen type I to type IV matrix components.[35] This also argues for a dominant role for the extracellular matrix in regulating cell activity and supports the view that type IV collagen is an anti-angiogenic maturation factor for endothelial cells. It is clear, therefore, that a prerequisite for angiogenesis is degradation of basement membrane type IV collagen.

In contrast, type III collagen is associated with elastic, vascular tissue and is found in high relative concentration in healing wounds with active neovascularisation.[36] In this respect, an inhibitory activity in normal retina for retinal endothelial cell growth[37] – which is manifested in the presence of normal adult but not fetal serum, on most extracellular matrix proteins such as fibronectin, laminin and collagen – is lost when the cells are cultured on type III collagen (Fig. 4). These experiments serve to demonstrate the fine balance that exists between stimulatory and inhibitory growth factors and the nature of the extracellular matrix.

Other Matrix Molecules

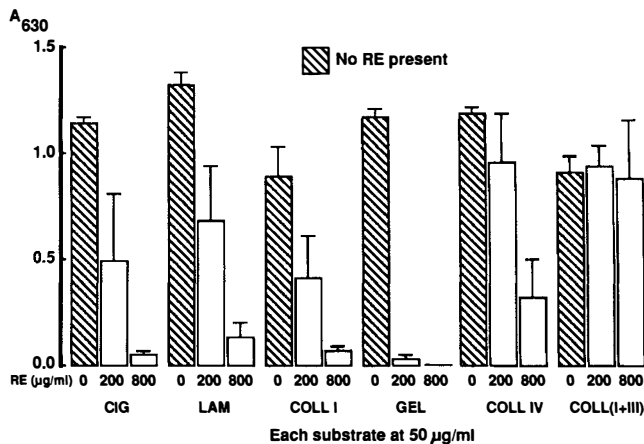Several other molecules are involved in the interactions

Fig. 4. *Effect of matrix components on retinal-extract (RE)-induced inhibition of serum-stimulated retinal endothelial cell (EC) growth. EC growth in platelet-depleted adult serum is inhibited if soluble extract of retina is present (see McIntosh et al.[37]). This effect is observed if the cells are grown in fibronectin (CIG), laminin (LAM), collagen types I and IV (COLL I, IV) and gelatin (GEL), but is lost if the cells are cultured on a matrix containing type III collagen (COL I+III). Cell proliferation was measured in a dye-binding assay as A_{630}.[37]*

between endothelial cells and the extracellular matrix. In the basement membrane, these include molecules such as the heparan sulphate proteoglycan (HSPG), perlecan,[38,39] thrombospondin, entactin, and vitronectin/plasminogen activator inhibitor-1 (PAI-1) (for review see Timpl[40]). In the interstitial matrix, during the invasive and/or migratory phase of the newly forming vessel wall cells, interactions with many proteins may occur such as other proteoglycans, fibrin, elastin and α2-macroglobulin. Extensive reviews on the structure and function of these molecules are available and only brief reference to their role in endothelial cell behaviour is made here.[40]

A low density form of HSPG appears to be an essential component of basement membranes,[41] where it probably acts as a storage site for growth factors such as fibroblast growth factor (FGF) (see later). HSPG probably has an inhibitory effect on normal endothelial cell growth.[42]

Thrombospondin is a large trimeric (450 kDa), widely distributed glycoprotein which regulates cell growth and migration and inhibits angiogenesis *in vivo*.[43,44] It is the most abundant protein in platelet α granules, from which it is released in the initial stages of tissue injury. It is also synthesised by endothelial cells and incorporated into the extracellular matrix. Transforming growth factor β (TGFβ) is tightly bound to thrombospondin in the α granules and it has been suggested that this accounts for the inhibitory effect of thrombospondin on endothelial cells. However, this question has yet to be settled.[45]

GROWTH FACTORS AFFECTING ANGIOGENESIS

The presence of soluble growth factors which might initiate retinal angiogenesis was suggested over 40 years ago.[4] 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.[46] 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 (Table II). Some of these factors are stimulatory (+) and others 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 (Table II). Certain factors such as tumour necrosis factor α (TNFα) may be stimulatory or inhibitory depending on the circumstances, while others act synergistically, such as insulin-like growth factor-I (IGF-I) and FGF, or in an integrated manner at different stages in the process of angiogenesis such as FGF, transforming growth factor β (TGFβ) and urokinase-type plasminogen activator (uPA). Some of these factors are discussed below.

Fibroblast Growth Factor (FGF), Transforming Growth Factor (TGF) and Plasminogen Activator/Plasminogen

FGFs belongs to a large family of heparin-binding growth factors which are widely distributed and account for a significant proportion of the angiogenic activity in soluble extracts of normal retinal tissue.[47] Currently at least seven isotypes of FGF are described of which two, acidic (aFGF) and basic (bFGF), have been sequenced and cloned.[48] bFGF is distributed in all tissues while aFGF is restricted to neural tissue including the retina.[49] FGFs are located in basement membranes bound to HSPG,[50] which protects them from proteolytic degradation[51] but also renders them inactive, possibly by inducing a conformational change in the molecule.[52] Since FGFs do not have a signal sequence, it is not clear how they are transported from the cell into the extracellular matrix; it has been suggested that active FGF is released from cells after injury or cell death, thereby allowing them to initiate the healing response.[53,54] However, it has also been proposed that FGF may be transported out of the cell by utilising the exocytosis pathway.[55]

FGFs are synthesised and secreted by many different cell types including fibroblasts, macrophages, endothelial cells and several neuronal cells.[56] The precise role of FGFs in diabetic retinopathy is unclear. In addition to being present in retinal vascular basement membranes, FGFs are present in photoreceptor cells and the interphotoreceptor matrix, where their role is more likely to be related to the physiology and turnover of retinal photoreceptor outer segments.[57] Recently FGF has been detected in the human diabetic retina but significantly no immunoreactive FGF activity was detected in association with proliferating retinal new vessels, only with non-proliferating mature vessels with thickened basement membranes.[58]

FGFs are stimulatory for many phases of the angiogenic response but their activity is closely linked to activity of other cytokines and proteases, particularly TGFβ and plasminogen activator. Transforming growth factors (TGFs) are multifunctional cytokines secreted by many cell types and carried in the circulation attached to specific

binding proteins.[59,60] TGFα has some pro-angiogenic activity[61] but TGFβ (of which there are at least six sub-types) is considered a major regulatory protein for many cellular processes, including angiogenesis. *In vitro* it is inhibitory for endothelial cell migration and growth,[62] but *in vivo* it promotes angiogenesis at the stage of vessel maturation.[63] Thus it seems to have a bi-functional role in new vessel formation.[64]

It has been suggested that regulation of endothelial cell proliferation is under pericyte control via activation of pericyte-secreted latent TGFβ, which in effect means proteolytic release of TGFβ from its binding protein.[65] This requires close contact between the endothelial cell and the pericyte.[66,67] The early loss of pericytes in diabetic retinopathy would therefore render the endothelial cells more susceptible to escape from growth control.

Pericyte loss, however, is not a prerequisite for retinal neovascularisation, as has been shown in an animal model of diabetes.[68] In addition, of the six currently known sub-types of TGFβ only TGFβ_1 is inhibitory for endothelial cells and even then only for certain types of endothelial cell.[64] Furthermore, TGFβ is produced by endothelial cells
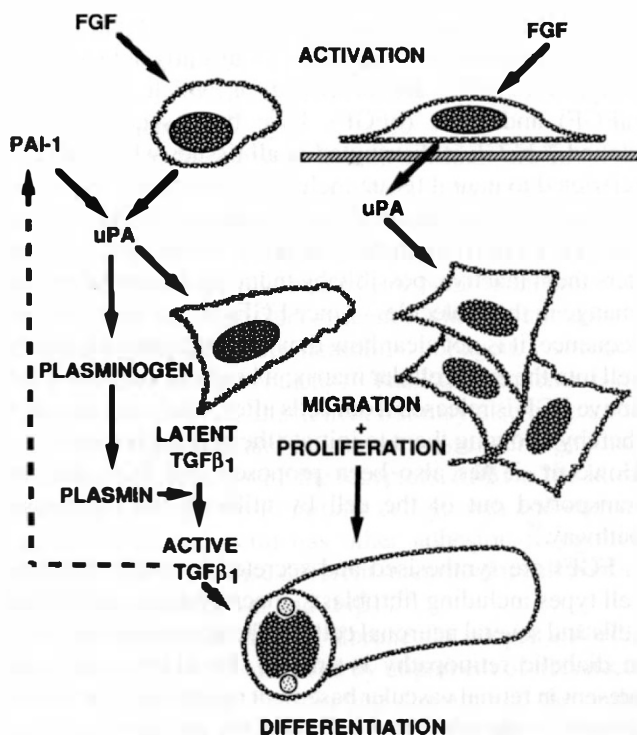
themselves[69] and it is likely that the angiogenic response is tightly regulated in an autocrine/paracrine manner via mechanisms involving plasminogen activation. According to this scheme, activation of the endothelial cell by an angiogenic growth factor such as FGF induces secretion of urokinase-type plasminogen activator (uPA) which directly stimulates migration of the cell via its specific receptor using the non-proteolytic portion of the molecule (Fig. 5).[70] Simultaneously, uPA converts extracellular matrix plasminogen to plasmin which in turn converts latent TGFβ to active TGFβ. This induces synthesis of plasminogen activator 1 (PAI-1) which inactivates the plasmin and switches off the cell activation signals. In the meantime, residual active TGFβ continues to act on the cell and to induce the cell to 'mature', i.e. to form capillary tubes and vessels (Fig. 5).[69]

It can be seen, then, that the balance that exists between stimulatory and inhibitory factors in endothelial cell activation is normally weighted in favour of growth inhibition, and anything that is likely to disturb the balance towards angiogenesis is rapidly counteracted by mechanisms designed to switch off the response.[37] These inhibitory mechanisms have to be disabled if a sustained angiogenic response is to occur.

Although many of the above studies have been performed in large vessel endothelial cells, there is compelling evidence that the mechanisms described are of a general nature. Thus retinal endothelial cells synthesise and respond to FGF and TGFβ and are activated to produce high levels of mRNA specific for uPA when cultured in the presence of diabetic serum (Fig. 6).
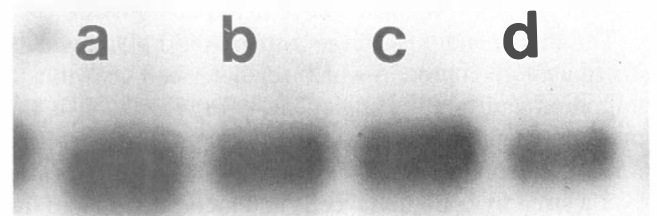

Fig. 5. *Diagram of proposed mechanism of FGF–TFGβ regulation of the angiogenic response. Initial activation of the endothelial cell by FGF leads to expression of plasminogen activator (uPA) on the cell surface, then permitting local pericellular degranulation of the matrix and forward movement, migration of the cell plus FGF-mediated proliferation. Simultaneous conversion of extracellular plasminogen to plasmin causes activation of latent TGFβ to active TGFβ which then induces production of plasminogen activator-1 (PAI-1), thereby inactivating uPA and switching off the angiogenic response. At the same time, active TGFβ induces differentiation of the newly migrated/divided endothelial cell to form capillaries and vessels. The extent of the 'angiogenic sequence' and thus the degree of vascularisation depends on the balance between the FGF/uPA system and the TGFβ/PAI-1 system.*

Fig. 6. *Northern hybridisation blot/autoradiograph of mRNA extracted from endothelial cells cultured in normal serum (d) and serum from patients with proliferative diabetic retinopathy (a, b, c), and probed with a uPA-specific cDNA probe. Increased uPA-specific mRNA is detectable in lanes a, b and c.*

Insulin, Insulin-Like Growth Factor (IGF-I) and Platelet-Derived Growth Factor (PDGF)

The discovery of FGF as the major heparin-binding growth factor in the retina generated considerable interest because it fitted well with the concept of a locally produced retinal factor which could be released after injury to the cell and its basement membrane, particularly ischaemia. However, FGF in vitro is a rather weak stimulator of endothelial cell growth, and is much more effective in the presence of serum, which suggests that circulating systemic co-factors may be important in the angiogenic response. Clinically, proliferative diabetic retinopathy (PDR) responds to changes in the level of circulating pituitary hormones, an observation which led to an investigation of IGF-I as a possible angiogenic factor in PDR.

IGF-I is a 7.6 kDa peptide, with about 70% homology to insulin and IGF-II, which together with PDGF, a 30 kDa dimer synthesised by several cell types, is considered to account for most of the growth promoting activity of serum in vitro for the majority of cell types. However, retinal endothelial cell growth is inhibited in normal clotted serum, perhaps due to the release from platelets of inhibitory factors such as platelet factor 4 (PF4), TGFβ and thrombospondin (Tables I and II).[71] In contrast, endothelial cells grow well in platelet-depleted serum (PDS),[30] indicating that PDGF is not a requirement for endothelial cell growth as it is for fibroblasts and smooth muscle cells.

As a result attention has focussed on the role of IGF-I in endothelial cell function. IGF-I is produced by several cell types but the major source is the liver. In vivo, it acts in a paracrine manner to mediate the activity of growth hormone. In experimental diabetes, impaired somatic growth has been attributed to diminished IGF-I production by the liver, with a concomitant increase in circulating binding proteins.[72] IGF-I is also produced by several other cell types including various retinal neuronal cells where it is presumed to have a role in retinal cell maturation.[73] IGF-I stimulates endothelial cell growth in vitro and competes with the insulin receptor.[74] IGF-I has also been reported to be chemotactic for endothelial cells.[75] IGF-I stimulates the expression and production of uPA by human retinal endothelial cells, suggesting that its mode of action in stimulating endothelial cell migration is similar to that of other growth factors such as FGF. Interestingly, the level of activator is increased in cells from diabetic patients.[76]

IGF-I circulates in the blood stream bound to its binding proteins, IGFBP-1 and -2,[77,78] and several studies have attempted to correlate IGF-I concentrations with severity of retinopathy. An early study concluded that there was a correlation between serum levels of IGF-I and degree of retinopathy,[79] although a second longitudinal study could not confirm these results but found that transiently elevated levels of IGF-I were detectable only at the time of active neovascularisation.[80] More recently, a large population-based study found that higher levels of IGF-I were associated with an increased frequency of severe retinopathy. Furthermore, significant concentrations of IGF-I[81]

have been detected in samples of vitreous fluid from diabetic patients following vitrectomy. It is therefore possible that IGF-I, produced both systemically and locally, could influence the development of new vessels either on its own or through synergistic interactions with other growth factors such as FGF and PDGF.

Other Growth Factors

There are many other molecules with angiogenesis-promoting activity (Table II). Some of these, such as interleukin-1 (IL-1),[82] are produced by a wide variety of cells, particularly when activated, while others such as interleukin-6 (IL-6)[83] and TNFα,[84] are produced by activated leucocytes and macrophages. Platelets also release a specific angiogenic factor which is active in vivo and in vitro, and has recently been described as having thymidine phosphorylase activity.[85] It therefore may act not directly on endothelial cells but via mechanisms involving the degradation products of thymidine. Recently, a tumour angiogenesis factor has been described with multiple effects on vascular permeability and endothelial cell growth: vasculotropin (also known as vascular permeability factor or vascular endothelial cell growth factor).[86] This factor has also been shown to induce mitogenesis in endothelial cells by a mechanism which appears to be independent of plasminogen activator.[87]

Retinal pigment epithelial cells synthesise and secrete several angiogenic cytokines including FGF, IL-1, TNFα, IGF-I and TGFβ, and may have a regulatory effect on endothelial cells via the plasminogen activator system.[88] However, other retinal cells are major sources of retinal growth factors, such as the photoreceptor cell.[89] In addition, FGF is found in high concentration in the interphotoreceptor matrix.[57] Currently, endogenous FGF and other heparin-binding growth factors are considered to be likely candidates for 'retinal angiogenic factor', but significant endothelial cell mitogenic activity remains after removal of heparin-binding factors from soluble retinal extract (Fig. 7).

Therefore, although the retina as a whole is a rich source of many growth factors, it is likely that the role of retinal growth factors under normal circumstances is to maintain the normal physiology of retinal cell function. When the retina is damaged as during ischaemia, some of this growth factor will be released locally but such limited amounts are unlikely to sustain an angiogenic response, particularly since powerful anti-angiogenic mechanisms are simultaneously activated. Indeed, this concept may partially explain why the vast majority of retinal angiogenic responses are arrested at the stage of the microaneurysm, which then undergoes involution and disappears. An alternative mechanism must be sought to explain the sustained and occasionally aggressive proliferative response of endothelial cells in diabetic neovascularisation.

PATHOGENESIS OF RETINAL ISCHAEMIA AND NEOVASCULARISATION

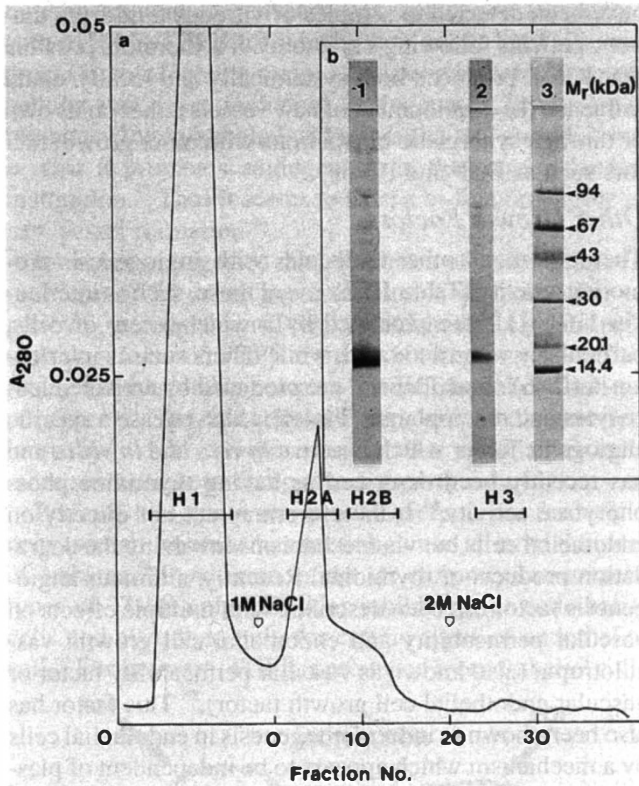While matrix factors and growth factors are agents for dif-

Fig. 7. *Protein absorption A_{280} spectrum of retinal extract after passage through a heparin–sepharose column to remove FGF and other heparin-binding growth factors (shown in inset on an SDS-PAGE gel with molecular weight standards). Fraction H1 (i.e. the eluate after removal of FGF) was collected and tested on endothelial cell cultures, revealing significant stimulatory growth activity. Fractions H2A and H2B contained FGF as shown by SDS-PAGE (inset, tracks 1 and 2).*

ferent aspects of the neovascular response, retinal ischaemia remains the generally accepted initiator of the entire process. Capillary closure is the direct cause of tissue ischaemia and this has been attributed to microvascular endothelial cell damage through mechanisms such as polyol accumulation, glycoxidation or NADH consumption (for reviews see Forrester[3,6]). Damaged endothelium then loses its non-thrombogenic surface as evidenced by a decrease in tissue plasminogen activator activity and a reduction in plasma fibrinolytic activity. The already hyperaggregable platelets[90] may then be induced to undergo spontaneous aggregation in the smallest vessels and lead to capillary closure and release of platelet factors which promote angiogenesis.

It is probable that such events occur in the diabetic eye but they are unlikely to lead to a sustained angiogenic stimulus because of the limited stores of growth factors which can be released from a non-renewable source such as aggregated platelets within a microvessel. A persistent stimulus would require live, and probably proliferating, cells to produce sufficient amounts of growth factor to overcome the natural growth-inhibitory mechanisms described above.

Recently, it has been reported that activated granulocytes and monocytes were the main cellular elements

responsible for widespread retinal capillary occlusion in a rat model of alloxan-induced diabetes.[91] In addition, foci of intraretinal neovascularisation in the later stages of the disease were characterised by diapedesis of monocytes and intraretinal proliferation of macrophages (Fig. 8). Accordingly, a model was proposed whereby systemic activation of leucocytes in the diabetic state induced increased adhesiveness in these cells for the endothelial cell, which led to capillary closure. Capillary obstruction occurred almost exclusively in the lower capillary layer, which in the rat is closely connected to the venules, and therefore presumably is a site of major decrease in vessel wall shear stress and preferential leucocyte adhesion, as observed in many other organs. Leucocyte endothelial cell adhesion in the pre-venule capillary was followed by diapedesis of mononuclear cells and proliferation/differentiation of these cells into macrophages in the tissues. Macrophages have long been recognised to play an important role in angiogenesis in other systems[92] and are a major cellular constituent of the pathology of diabetic retinopathy in humans (see above). As a result, in areas of capillary closure, a constant source of growth factor would be available, at least for the life-span of the macrophage, which if sufficiently prolonged might generate an angiogenic response in the remaining healthy endothelial cells.
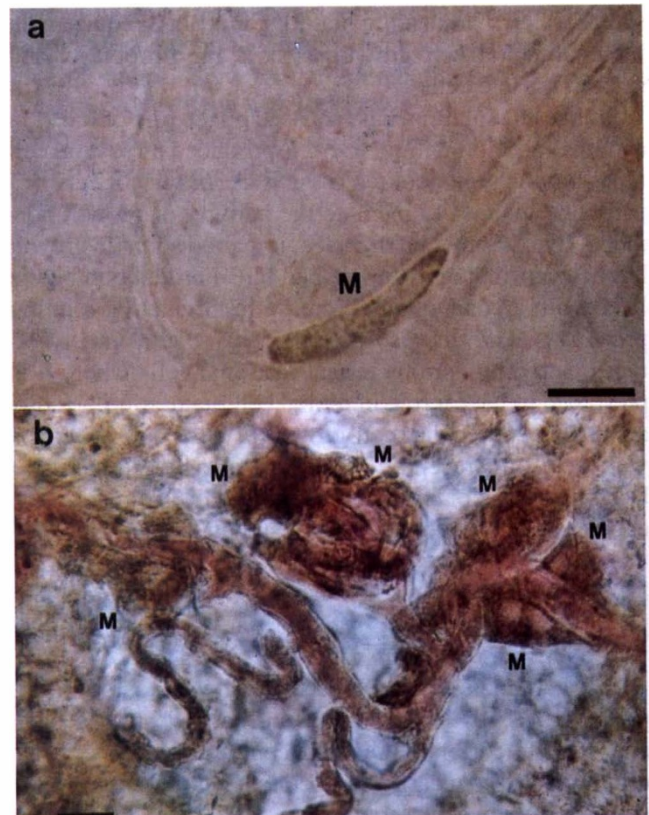

Fig. 8. *Retinal whole-mount preparations from rat with alloxan-induced diabetes (see Schröder et al.[91]). (a) Perfusion-fixed retinal whole-mount showing single monocyte (M) occluding a small retinal vessel. Scale bar represents 10 μm. (b) Non-perfusion-fixed retinal whole-mount showing intraretinal proliferation of small vessels with loops and dilations in close association with monocytic tissue infiltration (M). Scale bar represents 20 μm.*

We have also previously emphasised the potential role of endogenous macrophage (microglial cell) activation in an explant model of retinal angiogenesis.[93] In this model, new vessel outgrowth was consistently accompanied by macrophages, while in the prodromal phase of widespread retinal ischaemia spontaneous intraretinal macrophage activation was observed. Macrophages produce a very wide range of growth factors and also some endothelial cell growth inhibitors (in addition to those described above)[94] and it is likely that heterogeneity in the macrophage population will have a significant bearing on the microenvironment which leads to an angiogenic response. In spite of this, we propose that in diabetes a systemically activated leucocyte/macrophage-mediated mechanism is a more plausible model to explain capillary closure, retinal ischaemia and the resultant angiogenic response than existing models which rely predominantly on local production of angiogenic factor by damaged retinal cells.

CONCLUSION

Proliferative diabetic retinopathy is a complex condition which results from a combination of local and systemic factors. In the currently proposed model, systemic activation of leucocytes, particularly monocytes, leads to capillary closure through increased adhesiveness of the leucocytes to the damaged endothelial cell surface. Activated macrophages in the tissues, probably derived both from a circulating pool of cells[91] and from a resident microglial population,[93] provide a rich source of growth factors and other molecules such as matrix-modifying enzymes which lead ultimately to an angiogenic response in surrounding healthy endothelial cells.

Key words: Angiogenesis, Diabetic retinopathy, Growth factor, Matrix, Neovascularisation.

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