PERSPECTIVE

Role of vascular permeability factor/vascular endothelial growth factor in eye disease

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The eye contains highly vascularised and completely avascular tissues in close apposition. This specialised anatomy requires tight regulation of the balance between vascular quiescence and vascular growth. Growth normally occurs in ocular embryonic development, but is virtually absent from the eye in adult life.[1] In eye diseases associated with angiogenesis, this delicate balance is disturbed.[1–6] Angiogenesis plays a crucial role in disorders responsible for most blind registration in the Western world—that is, diabetic retinopathy (DR), retinopathy of prematurity (ROP), age-related macular degeneration (AMD), and a large number of other eye conditions.[1 5] Because of its importance in wound healing, tumour growth, and other pathological situations, angiogenesis has been extensively studied in the fields of oncology, rheumatology, cardiology, and ophthalmology.[7 8] The main interest in these efforts has been the notion that inhibiting angiogenesis may influence the course of tumour growth or other disease.[9 10] In ophthalmology, inhibition of angiogenesis may also prove to be of great value.[1 4–6 9]

Angiogenesis is a tightly controlled process which involves both endothelial cells and pericytes, and is influenced by numerous agonist growth factors, inhibiting factors, and extracellular matrix proteins.[7 8 11–14] Its importance in embryonic retinal vascularisation and eye disease (Fig 1) has long been recognised, and it was as early as 1948 that Michaelson suggested that it is the avascular fetal retina itself that produces a diffusible 'biochemical factor' capable of inducing vascular ingrowth, the production of which is associated with retinal metabolism.[15] Later, when the association of neovascularisation with retinal non-perfusion and ischaemia in pathological conditions such as diabetic retinopathy was recognised, Ashton and colleagues suggested that hypoxia may be the impetus for the production of this presumed factor.[16 17]

In recent years, an important candidate for Michaelson's 'factor' has emerged: vascular permeability factor or vascular endothelial growth factor (VPF/VEGF).[4 18–21]

Here we aim to describe exciting evidence pointing to the role this growth factor may play in eye disease. The understanding of the mechanisms of ocular angiogenesis derived from this work may lead to new therapeutic options for many common eye conditions.

VEGF is a major angiogenesis and vascular permeability factor

DISCOVERY OF VPF/VEGF

In 1982 the name vascular endothelial growth factor was first used to denote endothelium specific mitogenic activity isolated from calf retina.[22] Although this growth factor had similarities with the molecule presently known as VPF/VEGF, it was incompletely characterised.[22 23] VPF/VEGF was isolated in 1983 by Senger *et al* as the result of a search
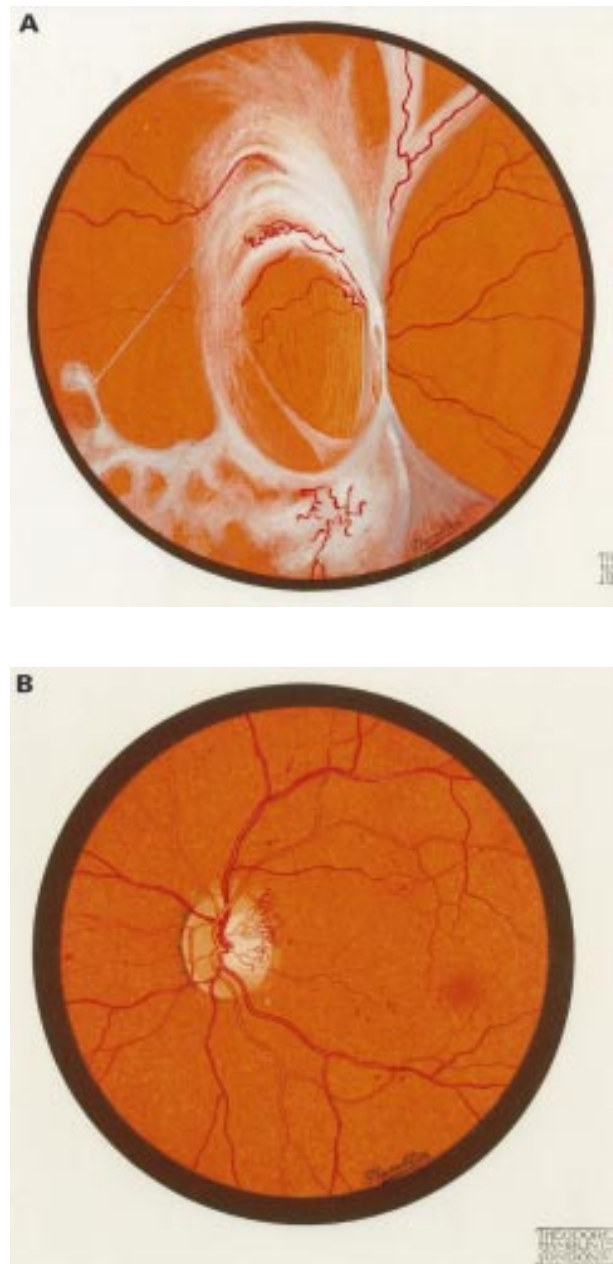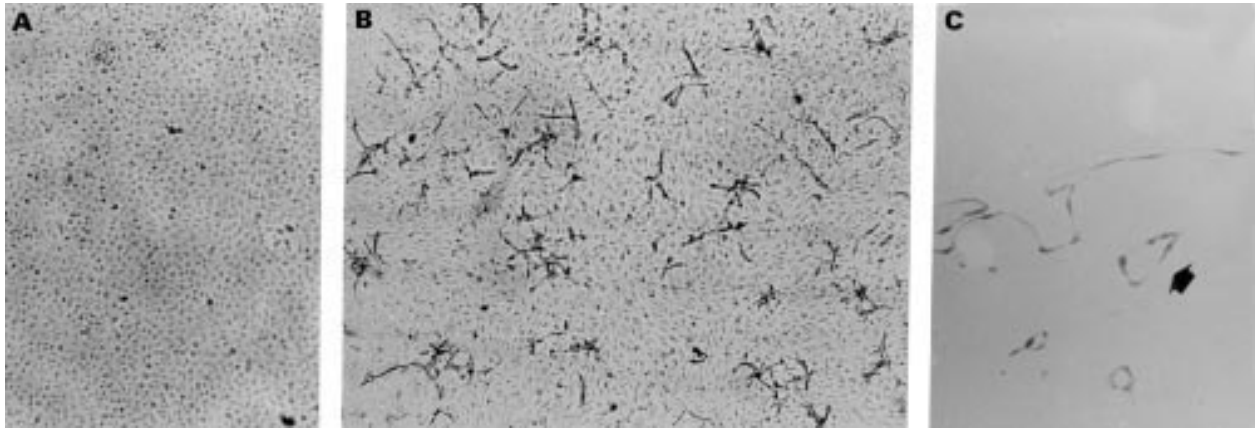

Figure 1 Early clinical observations established the importance of angiogenesis in eye disease: 'Retinitis proliferans' (proliferative retinopathy) in an 18-year-old female (A) and 'New vessels in vitreous', associated with retinal haemorrhages (B), as painted in 1929. From the archives of Moorfields Eye Hospital.

Figure 2   Formation of capillary-like tubular structures by cultured human microvascular endothelial cells in a three dimensional fibrin matrix is induced by simultaneous addition of growth factors VEGF and bFGF and the cytokine TNFa. (A) and (B) Phase contrast photomicrograph of control cells (A) and stimulated cells (B) (magnification × 30); (C) cross section through the top of the fibrin layer. Endothelial cells are visible on top of the fibrin and around lumina within the fibrin matrix (arrow) (× 340). (Courtesy Drs M Kroon and P Koolwijk.)

for tumour derived factors causing increased microvascular permeability.[24] These authors hypothesised that angiogenesis in tumours and healing wounds depends on a common pathway that involves the deposition of fibrin and other extravasated macromolecules in the stroma, rather than being initiated directly by angiogenetic factors.[24–26] The isolation of a tumour derived diffusible molecule enhancing this extravasation supported their hypothesis, hence the early name vascular permeability factor (VPF).[24] It took 6 years to recognise that this molecule, apart from its effect on microvascular permeability, effectively had all the properties of a true angiogenesis factor.[27 28] This coincided with the isolation and subsequent cloning of two 'new' endothelial mitogens named vascular endothelial growth factor (VEGF) and vasculotropin[29–33] which in the following years were recognised to be identical to VPF.[29 34 35] In this review we will adhere to the most commonly used designation for this factor: VEGF.

BIOCHEMICAL PROPERTIES
VEGF is a glycoprotein dimer of 34–45 kDa with some structural homology to platelet derived growth factor (PDGF), and close homology with placenta growth factor[18 36–40] and two other gene products, VEGF-B[41] and VEGF-C.[42 43] VEGF is a secreted factor in contrast with certain other highly angiogenic cytokines, such as acidic and basic fibroblast growth factor (aFGF, bFGF).[11 44–46] Many different cell types, including endothelial cells, produce VEGF in vitro.[47–49] Four different isoforms of VEGF (with 206, 189, 165, and 121 amino acids) arise from the same gene by alternative splicing.[25 50] The isoforms appear to have identical biological activity but differ in molecular weight and affinity for heparin which may affect their bioavailability in tissues.[51–53] Thus, the 121 and the predominant 165 species are soluble factors while the larger forms with higher heparin affinity are cell or extracellular matrix associated.[53] Enzymatic cleavage of these larger forms by plasmin can lead to the release of active diffusible VEGF and this may provide one mechanism by which VEGF activity is regulated in vivo.[53 54]

IN VIVO AND IN VITRO ACTIVITIES RELATED TO ANGIOGENESIS
In vivo, VEGF potently increases microvascular permeability of post capillary venules and capillaries[24 55 56] and can rapidly induce fenestrations in continuous endothelia when injected in skin or muscle.[57] This effect on endothelial permeability is probably involved in the pathogenesis of malignant ascites,[58] ovarian hyperstimulation syndrome,[59] oedema around brain tumours,[60 61] bullous skin diseases, and psoriasis.[62 63]

The extravasation of plasma proteins caused by VEGF may be a crucial step in the neovascular response.[12] However, VEGF has a number of other biological activities consistent with a role as a direct angiogenesis factor: it induces neovascularisation in several in vivo angiogenesis assays.[18 27 28 64 65] It is mitogenic for cultured vascular and lymphatic endothelial cells[27 56 66] and stimulates their migration and tube formation, the so called 'angiogenesis in vitro' (Fig 2).[67 68] It also enhances endothelial glucose transport,[69] and induces expression of interstitial collagenase, the urokinase-type and tissue-type plasminogen activators and the urokinase receptor in these cells.[70–72] Collagenases and plasminogen activators are proteases that enable migration of endothelial cells through the interstitium or a fibrinous exudate during angiogenesis.[70–72] In addition, VEGF induces the expression of $\alpha_v\beta_3$ integrin and osteopontin,[44] proteins involved in cell-matrix interaction and angiogenesis.[73–75] In conclusion, VEGF is able to stimulate all major functions of endothelial cells needed in angiogenesis—increased permeability, migration, proliferation, and tube formation.

THE VEGF RECEPTORS
Two high affinity receptors for VEGF, named VEGFR-1 or Flt-1 and VEGFR-2 or KDR (Flk-1 in mouse)[35 76–80] have been characterised. They are both transmembrane proteins with cytoplasmic tyrosine kinase domains,[80] and are expressed on endothelial cells, including retinal endothelial cells.[81] The two receptors appear to mediate different biological effects of VEGF.[80]

VEGF receptors are upregulated in vivo in the microvasculature in conditions with overexpression of VEGF.[19 63 82–87] In vitro, hypoxia can induce endothelial VEGF receptor expression, possibly by a paracrine mechanism.[88] In cultured retinal endothelial cells, hypoxia initially downregulates VEGFR-2/KDR via adenosine and the adenosine A2 receptor, while chronic hypoxia leads to increased VEGFR-2 expression.[81] On the other hand, transforming growth factor β downregulates the expression of the VEGFR-2 in cultured endothelial cells.[89]

The VEGF receptors and specifically VEGFR-1/Flt-1 have been demonstrated in many other cell types, including retinal glia, retinal pigment epithelial cells, monocytes, mast cells, corneal endothelial cells, and cultured pericytes.[19 90–103] In most of these cell types, the growth factor stimulates migration through binding to VEGFR-1/Flt-1, but not proliferation.[90 92 97 99 100] Thus, VEGF may help to recruit pericytes, mast cells, monocytes, and other cells during angiogenesis or inflammation.

The mitogenic effect of VEGF, which is mediated in cultured endothelial cells through binding to the VEGFR-2/KDR receptor, is restricted to endothelial cells in vivo and to endothelial cells, bovine retinal pericytes,[98] and some epithelial cell types in vitro, including retinal pigment epithelial cells.[101–103]

ROLE OF VEGF AND ITS RECEPTORS IN EMBRYONAL DEVELOPMENT

Recent evidence shows that VEGF plays a major role in the development of the vascular system in embryogenesis.[8 104–107] In early embryos, VEGFR-2 is expressed in mesodermal cells giving rise to angioblasts, and in later stages both VEGF and its two receptors are highly expressed in a distribution suggesting a paracrine mechanism of vascular development induction.[18 108–110] For example, in the development of brain and retina, expression of VEGF correlates temporally and spatially with the ingrowth of capillaries, consistent with Michaelson's hypothesis of a retina derived factor.[15 104 106] In the developing retina, VEGF may also act as a survival factor for endothelium.[111 112] The two VEGF receptors appear to mediate distinct functions in this process as mutations in the two receptor genes show differential lethal developmental abnormalities in transgenic mice.[113 114] Mice lacking functional VEGFR-2/KDR fail to develop blood islands and show no blood vessel development,[113] while embryos deficient in VEGFR-1/Flt-1 form endothelial cells with normal differentiation but abnormal assembly of the cells in disorganised blood vessels.[114] This may be consistent with a role of VEGFR-2 in mediating a mitogenic/differentiation response of VEGF on embryonic endothelial cells and an effect on migration of vascular cells via VEGFR-1.

Mice embryos lacking one allele encoding for VEGF also have abnormal vascular development and die in utero, suggesting a tight dose dependent regulation of embryonic vascular development by VEGF.[115 116]

INTERACTIONS WITH OTHER GROWTH FACTORS

Other growth factors and cytokines can also potently induce or inhibit angiogenesis.[7 9–13 44 46] The interactions and stimulatory and inhibitory actions of these factors on cells in vitro and in vivo vary in different conditions and tissues.[46 68 117 118] For example, whereas VEGF alone induces capillary-like tubular structures of bovine endothelial cells in a fibrin matrix in vitro,[67] it requires the simultaneous presence of tumour necrosis factor α (TNF-α) to induce human microvascular endothelial cells to form such structures (Fig 2).[68] Both in vitro and in vivo, VEGF and bFGF synergistically enhance angiogenesis.[12 67 68 119–121] Furthermore, many growth factors/cytokines can affect angiogenesis and microvascular permeability indirectly through the upregulation of VEGF.[12] In vitro, bFGF, EGF, IGF-1, PGE$_2$, TNF-α, PDGF-BB, angiotensin II, and TGF-β all upregulate VEGF expression in different cell types—for example, smooth muscle cells and fibroblasts.[11 12 122–129]

From all these observations the notion has derived that the actions of a specific cytokine are contextual,[12 118] in that they are dependent on the presence of other growth factors and inhibitors, extracellular matrix composition, and expression of specific receptors on the target cells.[7 12 13 46 130]

HOUSEKEEPING FUNCTIONS OF VEGF IN NORMAL ADULT TISSUES

It is important to realise that the functions of VEGF are not limited to angiogenesis, wound healing, and embryogenesis.[8] VEGF messenger RNA has been demonstrated in many normal adult tissues without angiogenesis, in mouse, rat, and humans, with highest levels in lung, liver, kidney, heart, and adrenal gland.[106 131–133] This suggests unknown housekeeping functions of the cytokine.[106 131–133] Based on expression of VEGF in glomeruli and collecting duct epithelium of the kidney,[108 134 135] and its receptors in endothelium and mesangial cells of the glomerulus,[95] it was proposed that the factor may induce baseline vascular permeability and/or the fenestrated endothelial phenotype.[95 108 134 135] In liver and choroid plexus in the brain a similar mechanism may occur.[106 136] The observation that exogenous VEGF can rapidly induce fenestrations in the continuous endothelium of skin and muscle in vivo seems to support this hypothesis.[57] Other suggested functions of VEGF, which act via endothelial cell stimulation, are in the response of cardiac and skeletal muscle to stimulation and hypoxia,[137–141] coronary artery relaxation,[142] and pancreatic islet maintenance.[91 143] It may also act as an epithelial mitogen in hair formation.[102] Furthermore, VEGF plays a role in monocyte migration[92 97] and haematopoiesis, possibly as a survival factor for certain haematopoietic cells, including leukaemia cell lines.[93 144]

Role of VEGF in hypoxia induced angiogenesis

ROLE OF VEGF IN PHYSIOLOGICAL AND PATHOLOGICAL ANGIOGENESIS

VEGF and its receptors are overexpressed in many tissues with blood vessel growth, often together with other angiogenesis factors.[12 19 63 82–86 107 145 146] Angiogenesis in the cycle of the female reproductive organs correlates with VEGF expression,[147–150] and is possibly under hormonal control.[147 149] Angiogenesis is also associated with upregulation of VEGF messenger RNA in tissue responses to injury, such as wound healing[86] and formation of collateral vascularisation in cardiac ischaemia,[139–141] and in pathological angiogenesis in solid tumour growth,[19 45 82 85 133 145] rheumatoid arthritis,[151] psoriasis,[63] capillary haemangiomas,[152 153] and atherosclerosis.[154]

In many human and experimental tumours VEGF expression correlates with tumour vascularity, tumour progression, and metastatic potential.[83 84 155–158] The marked effect on the growth of certain experimental tumours in vivo by inhibition of VEGF by neutralising antibodies[159–161] or transfection of antisense VEGF cDNA,[162] and by inhibition of its receptors by transfection of a dominant negative VEGFR-1/Flk-1 mutation[163 164] further underscores the importance of this growth factor in tumour angiogenesis. Therapeutic inhibition of VEGF may therefore prove a valuable new treatment modality in cancer patients.

VEGF IS UPREGULATED BY HYPOXIA IN VITRO AND IN VIVO

In 1992, Shweiki et al showed that VEGF messenger RNA was mainly expressed in the vicinity of necrotic and presumably hypoxic foci in glioblastoma multiforme and they demonstrated that hypoxia upregulated VEGF expression in glioma cells in vitro.[145] Since then other tumour cells,[165 166] vascular smooth muscle cells,[122 123 126] pericytes,[167 168] retinal pigment epithelial cells,[167] and endothelial cells[96 167–170] were found to have de novo (endothelial cells), or upregulated (other cell types) expression of VEGF when grown under hypoxic conditions.[171] In vascular smooth muscle cells this effect is synergistically enhanced by bFGF or PDGF-BB.[123 126] In multicell spheroids of glioma cultured in vitro, VEGF expression is found in the inner hypoxic cells.[172–174]

It is not entirely clear how hypoxia upregulates VEGF at the molecular level.[173 175] This appears to be partly due to transcriptional activation of the VEGF gene, in a manner similar to hypoxia induced erythropoietin gene expression,[169 171 176–181] but mostly by post-transcriptional

stabilisation of VEGF messenger RNA.[165 169 173 182–185] Furthermore, adenosine, which is released from hypoxic tissues, can upregulate VEGF expression[166] through the adenosine A(2a) receptor and the cAMP dependent protein kinase A pathway, as has been demonstrated in retinal vascular cells.[168]

In addition to hypoxia and low glucose, mutations in the *p53* cancer suppressor gene[186 187] and the von Hippel Lindau tumour suppressor gene,[185 188] or activation of the *ras* oncogene may also induce upregulation of VEGF expression in human cancer cells, providing a possible mechanism of the neoplastic progression associated with these genes.[175 185–189]

Does VEGF play a role in eye disease?

OCULAR ANGIOGENESIS

Angiogenesis is crucial in the development of the eye as well as in the pathogenesis of a variety of ocular diseases.[1–6 104] Corneal neovascularisation, rubeosis of the iris, proliferative retinopathies, and subretinal neovascularisation may result from a multitude of unrelated disease states. It should be realised that in all these conditions vascular growth is part of a wound healing response which usually also involves inflammatory cells, (myo)fibroblasts, and in the posterior segment, glial cells or activated pigment epithelial cells.[11 12] In the past three years, extensive evidence has become available that VEGF is involved in probably all forms of ocular angiogenesis. In fact, it corresponds well with the diffusible retinal vasoformative 'factor' as originally proposed by Michaelson in 1948.[15] However, the available data imply that other factors also contribute to the initiation or maintenance of ocular neovascularisation.

VEGF AND THE CORNEA

Exogenous VEGF potently stimulates angiogenesis in the cornea of experimental animals with no or little inflammatory response.[65 190] However, the role of the factor in pathological corneal neovascularisation, as occurs in contact lens wearers, after corneal trauma and transplantation or in inflammatory conditions, is unclear. Recently it has been demonstrated that VEGF is produced in whole rabbit corneas kept in culture, and is upregulated in the rat cornea in vivo after 2 weeks of atmospheric hypoxia.[191 192] Epithelial cells from normal avascular cornea produce VEGF, but do so at a lower level than epithelial cells from vascularised cornea or conjunctiva.[193] These preliminary data suggest some role for the factor in corneal angiogenesis. Another interesting finding is the expression of the Flt-1/VEGFR-1 and VEGF in dedifferentiated corneal endothelial cells in vitro[194] and the migration, but not proliferation, of these cells in response to VEGF.[99]

RETINAL ISCHAEMIA

In clinical ophthalmology it is evident that diseases of the retina which are associated with impaired capillary or venous circulation can lead to retinal neovascularisation (proliferative retinopathies) (Figs 1 and 3).[1–6 104 195] Viable ischaemic retinal tissue seems to be a prerequisite for an angiogenic response in these conditions, probably through the release of an angiogenic factor by retinal cells triggered by hypoxia.[15–17 195 196] Oxygen measurements in experimental animals and patients indeed have shown that retinal Po_2 is very low in ischaemic retinal conditions.[6 197] Surprisingly, panretinal photocoagulation, which is an effective treatment modality for proliferative retinopathies, can restore retinal Po_2 levels, most probably through a decrease of outer retinal oxygen consumption and improved oxygen diffusion from the choroid.[197]
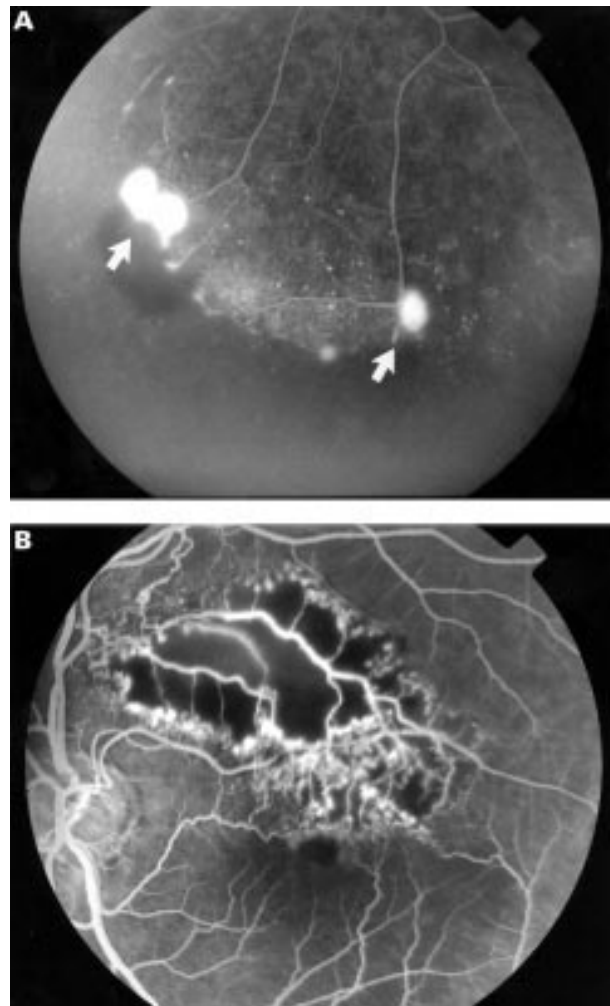

Figure 3 Retinal non-perfusion (recognisable as dark areas) in two patients with inflammatory vasculitis (A) and branch retinal vein occlusion (B). Note the profuse leakage of newly formed vessels (arrows) on the edge of the peripheral retinal non-perfusion (A) and the leakage of dilated vessels in and on the edge of the non-perfused area in the macular retina in (B). Experimental evidence suggests that these vascular changes may (in part) be mediated by VEGF.

Strong evidence now suggests that VEGF is a major cytokine produced by hypoxic retina. In a primate model of retinal ischaemia produced by branch vein occlusions it was shown that the resulting vascular dilatation, leakage, and endothelial proliferation in the iris temporally correlate with upregulated expression of VEGF messenger RNA in the ischaemic retina, and with elevated levels of growth factor in aqueous.[198] A neutralising antibody to VEGF injected into these eyes prevented the vascular changes in the iris, but had no effect on the retinal production of the growth factor, indicating that VEGF is indeed required for the observed effect on the iris.[199] In this model, scatter photocoagulation led to recovery from retinal hypoxia, and a decrease of VEGF expression in the retinal areas treated with laser.[200] Comparable results have been obtained in other experimental models of retinal ischaemia in rabbits and rats.[201 202] These studies suggest that VEGF plays a key role in ischaemia induced permeability and angiogenesis.

Several cell types, including ganglion cells, appear to express VEGF in the ischaemic retina in vivo.[203] These findings correlate with in vitro experiments that have shown that retinal pericytes,[167 168] endothelial cells,[167 168] and glial cells[204–206] produce VEGF in response to hypoxia.[104 167 168 205 206] Possible mechanisms for increased

VEGF production in ischaemic retina in vivo are by a paracrine effect of increased levels of adenosine and by hypoxia induced VEGF messenger RNA stabilisation[168 182] (see earlier).

The conclusions from the animal studies cited above are supported by recent reports showing that recombinant VEGF injected into the vitreous of monkeys is sufficient to induce vascular changes in the iris and eventually neovascularisation and neovascular glaucoma.[207 208] In the retina itself, intravitreal VEGF induces vascular leakage, hyperplasia, and the formation of microaneurysms in monkeys[209] and formation of preretinal new vessels in rabbits.[208] Remarkably, in these models, the presence of exogenous or upregulated endogenous VEGF is always associated with marked early effects on vascular permeability,[210] but does not necessarily lead to angiogenesis. The typical preretinal neovascularisation as seen by clinicians in the posterior segment of patients appears only late in VEGF injected rabbits and not at all in monkeys.[198 199 201 208 209] These findings suggest that other factors are important and necessary to lead to true retinal neovascularisation.

This is further emphasised by recent work indicating that VEGF and its receptors may also be involved in retinal conditions that are not associated with angiogenesis or ischaemia. Expression of VEGF and both its receptors was found in cultured human RPE and glial cells at the RNA level,[94 101] and immunohistochemical VEGF staining was found to be increased in situations where breakdown of the blood-retinal barrier is prominent such as experimental autoimmune uveitis in rats, human uveitis, and aphakic/pseudophakic macular oedema.[211]

ROLE OF VEGF IN DIABETIC RETINOPATHY AND VENOUS OCCLUSIVE DISEASE

Diabetic retinopathy (DR) is characterised by the development of microvascular leakage and focal areas of retinal ischaemia in non-proliferative DR (NPDR), or by neovascularisation originating from the disc and peripheral retina in response to widespread retinal ischaemia (proliferative DR).

Retinal vein occlusions may also show extensive retinal non-perfusion with leakage and ischaemia (Fig 3B) leading to retinal or iris neovascularisation.[2 3 212–214] Based on the previously described findings in animal models it is not surprising that upregulation of VEGF messenger RNA, and marked immunohistochemical microvascular staining for the factor have been demonstrated in the retinas of patients with proliferative DR and central retinal vein occlusions (CRVO).[201 215 216] Furthermore, several studies have demonstrated significantly elevated levels of VEGF in ocular fluids from patients with active diabetic proliferative disease or ischaemic CRVO compared with patients with inactive proliferative disease, after extensive laser surgery, or those with NPDR.[217–219] None of the other known angiogenesis factors, except insulin-like growth factor-1 (IGF-1),[44 129 220] show such a strong correlation with ischaemia related ocular angiogenesis in humans.[217–219 221] In addition to hypoxia, elevated glucose levels alone may also upregulate VEGF in diabetics.[222] However, it is remarkable that a significant number of diabetic patients with established active neovascularisation have undetectable levels of VEGF in ocular fluids.[217–219] Although this observation does not exclude the possibility that VEGF plays a primary role in the onset of retinal angiogenesis, possibly when widespread retinal ischaemia leads to a certain critical level of the growth factor in the retina or vitreous, it does indicate that other factors also can contribute to active neovascularisation in the eye.

More localised retinal expression of VEGF may only lead to increased microvascular permeability as recent work suggest that VEGF is also upregulated in NPDR and other causes of retinal oedema.[211 215 223 224] Surprisingly, increased immunohistochemical staining for VEGF could even be demonstrated in glial cells in the retina of patients with diabetes without signs of DR.[224] Still, in diabetic rats, increased microvascular permeability, as demonstrated by extravasation of endogenous albumin, correlates with elevated expression of VEGF messenger RNA and immunohistochemical demonstration of the growth factor in microvessels.[223 225] Retinal microvascular leakage is a major cause of visual loss in NPDR, vein occlusions, and other conditions and the above results suggest that VEGF inhibition may be used to treat these disorders.

RUBEOSIS OF THE IRIS

Growth of leaky new blood vessels on the surface of the iris is a feared complication of uncontrolled proliferative DR, ischaemic CRVO, and tumours of the posterior segment of the eye.[226] In particular, neovascularisation of the chamber angle with contraction of scar tissue may occur and lead to neovascular glaucoma and, potentially, loss of the eye. Rubeosis has long been attributed to diffusion of a vasoformative factor from ischaemic posterior segment structures.[2 212]

Recent reports indicate that VEGF corresponds well with this factor.[218 221] In a series of 38 patients with iris neovascularisation 85% of the cases with active rubeosis had detectable levels of VEGF in the aqueous or vitreous fluid, against only one out of five (20%) cases with inactive iris neovascularisation.[218] This difference was statistically significant. Another study demonstrated increased microvascular immunohistochemical staining for VEGF in the iris and other ocular structures of enucleated eyes with choroidal melanoma, but did not mention a relation with rubeosis.[227] Furthermore, the experimental animal models described above show a convincing relation between retinal VEGF expression induced by ischaemia and increased vascular permeability, dilatation, and endothelial proliferation in iris vessels.[198 199 202] However, these changes occur much earlier after onset of experimental retinal ischaemia than rubeosis occurs in ischaemic CRVO seen clinically.[212 214] They have been interpreted to represent true iris neovascularisation.[198 199] However, they could just represent changes in pre-existing iris vessels,[208] and as such may be quite different from the sprouting new vessels on the surface of the iris seen in clinical rubeosis.

RETINOPATHY OF PREMATURITY

Morphological studies on the mechanism of the normal development of retinal vasculature, and on the pathogenesis of retinopathy of prematurity (ROP) helped to form the concept of a hypoxia induced retinal vasoformative factor as put forward by Michaelson and Ashton.[15–17 196] In the mammalian embryo, sprouting of vessels occurs from the hyaloid vessels at the disc towards the retinal periphery, preceded by mesenchymal cells and astrocytes.[15–17 196] Indeed, recent work has shown that in newborn rats and fetal kittens, early expression of VEGF by these astrocytes in the inner retina is closely followed by formation of the inner vascular network,[104 228 229] and that expression of the factor by Müller cells in a later stage precedes the ingrowth of blood vessels from the superficial network to the level of the inner nuclear layer and subsequent formation of the outer capillary plexus.[104] This pattern led these authors to propose that hypoxia caused by the onset of neuronal activity is the stimulus for strategically located astrocytes and Müller cells to produce VEGF at different stages of development, leading to horizontal or vertical vascular ingrowth, respectively.[104] When hypoxia is relieved by the new vessels, VEGF expression decreases and angiogenesis

comes to a halt.[104] This reconstruction is consistent with the concepts proposed by Michaelson and Ashton.[15 17]

Retinopathy of prematurity may be the result of a distortion of these processes.[104 111 196 204 230 231] This disease is characterised by the obliteration of newly formed retinal vasculature, and cessation of peripheral vascular outgrowth in the premature infant as a result of hyperoxia from artificial respiration with high oxygen levels. When the child returns to breathing room air, a neovascular response follows consisting of abnormal, leaky, preretinal vessels on the edge of the avascular tissue, which may invade the vitreous and can lead to tractional retinal detachment and blindness.[196 230] The role of VEGF in ROP has been extensively studied in experimental animal models of the disease. In these models, newborn rodents, pups, or kittens are first kept in hyperoxic (70–80% oxygen) conditions for 4–5 days, and are then returned to room air. In such a model in the rat, 2 days of hyperoxia led to downregulated expression of VEGF, and this preceded endothelial apoptosis and vascular closure.[111] These vascular effects could be prevented by a single intravitreal injection of VEGF before exposure to hyperoxia.[111] Similar results were obtained in an identical mouse model.[112] In these models, after return of the animals to room air, VEGF was markedly upregulated in the affected retinal areas, presumably in Müller cells, before preretinal and vitreal neovascularisation occurred.[204] Inhibition of VEGF in these mice significantly diminished the number of (presumed) endothelial cells in the vitreous, interpreted by these authors as a measure of retinal neovascularisation.[232 233] Surprisingly, in these rodent models vascular closure caused by hyperoxia occurs in the posterior retina, while the already fully formed peripheral vasculature remains unaffected,[111 204] the reverse pattern from human ROP. In the feline model of ROP developed by Ashton, which resembles human ROP more closely,[230] similar results were obtained.[231] In these kittens, overexpression of VEGF in peripheral avascular retina after return to room air was apparently situated in the ganglion cell layer and associated with degeneration of the astrocytes.[231] These findings indicate that VEGF, in addition to its function as an angiogenesis factor, may also act as a survival factor for newly formed capillaries in the developing retina and suggest an important role for VEGF in the pathogenesis of human ROP. However, the inability of VEGF antagonists to completely inhibit angiogenesis in the rodent ROP models suggests that VEGF is not the only factor involved in angiogenesis in ROP.[232 233]

RETINAL ANGIOMAS
Retinal capillary angiomas may occur in the context of von Hippel–Lindau (VHL) disease. In VHL haemangioblastomas from the brain, which are histologically indistinguishable from the retinal angiomas,[234] VEGF and its receptors were shown to be markedly upregulated.[152 153] The VHL tumour suppressor gene has been cloned and recent work suggests that the VHL protein is involved in the oxygen dependent regulation of production of VEGF, platelet derived growth factor B chain and the GLUT-1 glucose transporter at the post-transcriptional level.[185 188 235] Therefore, inactivation of the VHL tumour suppressor gene, as found in VHL disease, may lead to uncontrolled production of VEGF.[185 188 235] Though not yet demonstrated for retinal angioma, the possibility exists that this mechanism is involved in the pathogenesis of retinal angiomas in VHL disease.

CHOROIDAL NEOVASCULARISATION
Subretinal or choroidal neovascularisation (CNV) is a major complication of diseases affecting the retinal pigment epithelium (RPE)-choroid interface, most notably age-related macular degeneration (AMD). It is characterised by the growth of a fibrovascular tissue derived from the choroid. Macrophages, myofibroblasts, and transdifferentiated pigment epithelial cells accompany the growing blood vessels, leading to the formation of a fibrotic scar. Several angiogenic and other growth factors have been found in these membranes,[11] and a role for VEGF in this common pathway of subretinal angiogenesis has been suggested by recent experimental data. The growth factor has been shown by immunohistochemistry in macrophages,[236] transdifferentiated RPE,[237] and by in situ hybridisation in fibroblasts and inflammatory cells[238] in surgically removed specimens of these membranes, and in the RPE and retina of postmortem eyes of patients with age-related maculopathy with or without CNV.[224 239] These observations are supported by the expression of VEGF by cultured RPE cells and choroidal fibroblasts.[101 240 241] Furthermore, significantly elevated levels of VEGF were found in the vitreous of patients with CNV.[242] How all these different observations fit together to explain the pathogenesis of CNV remains unclear.

POSSIBLE HOUSEKEEPING FUNCTIONS OF VEGF IN THE NORMAL EYE
As in other adult tissues, low constitutive expression of VEGF messenger RNA has been demonstrated in almost all tissues of the normal eye, most notably in the ciliary body, conjunctiva, RPE/choroid, and lens.[111 203 243 244] In vitro, RPE cells produce VEGF,[101 167 240 245] and in a coculture system this factor is partly responsible for enhanced choroidal endothelial tube formation induced by overlying RPE cells.[245] Therefore, it has been hypothesised that RPE derived VEGF may act as a trophic factor for the choriocapillaris.[11 240] In general, however, the function of VEGF expression in the normal eye is unknown.[243]

VEGF and its receptors as targets in ocular therapy
CLINICAL CONSIDERATIONS IN VEGF INHIBITION
Despite considerable therapeutic advances consequent upon the introduction of laser treatments, pathological angiogenesis and increased vascular permeability are still major causes of visual loss.[4 5 6] Laser treatment is often unpleasant for the patient and its therapeutic effect depends on ablation of viable normal tissue, therefore leading to a loss of function.[197] VEGF has been implicated in the pathogenesis of all the common conditions where laser treatment is currently used: NPDR and proliferative DR, RVO, ROP, and CNV. Inhibition of VEGF activity in these patients may prove a valuable alternative or additional therapeutic option and this could mean a major advance in ophthalmology.

From the previous paragraphs it is reasonable to conclude that VEGF plays an important and probably a pivotal role in ocular angiogenesis and increased microvascular permeability. Whether this role is mainly in initiation (directly or indirectly) or in maintenance of a neovascular response and/or vascular leakage is not known. This would have important implications for the use of VEGF inhibitory agents, as a significant number of patients still present with more or less established neovascularisation or macular oedema. Preventive neutralisation of VEGF in the experimental rodent models of ROP[232 233] or in the cynomolgus monkey venous occlusion model[199] has a significant inhibitory effect on preretinal neovascularisation or iris vascular changes, respectively. However, it remains to be seen how ocular VEGF inhibition will affect active or advanced neovascularisation or established oedema.

In the past 25 years several indications for laser therapy in DR and vein occlusions have been established through carefully designed randomised clinical trials. It seems reasonable to use these indications as starting points for planning clinical trials with VEGF inhibitory agents. Initial trials should use such agents only in situations that are known not to benefit from laser or in cases that have not responded to laser therapy, as it would be unethical to deny patients the established beneficial effect of the present laser treatments. Subsequent trials may evaluate their use as adjuvants or replacements for laser therapy.

THERAPEUTIC USE OF VEGF

Therapeutic use of VEGF could also be useful in certain situations, such as in stimulating collateral formation in limb ischaemia resulting from arterial occlusion.[246][247] In the developing retina VEGF appears to act as a survival factor for the microvasculature, so that exogenous VEGF may be able to prevent vascular obliteration in ROP.[111][112]

STRATEGIES IN ANTI-VEGF THERAPY

The actions of VEGF may be inhibited in several ways. Which of these approaches is clinically useful remains to be determined, as all are still in an experimental stage of development. Some proposed strategies interfere with the basal or hypoxia induced production of VEGF by effector cells. Others are aimed at neutralising soluble VEGF or to prevent its actions by blocking receptors or the transduction of receptor mediated intracellular pathways in target cells. Finally, VEGF induced angiogenesis may be inhibited by interference with the microvascular cellular response to the growth factor.

Inhibition of VEGF production

Antisense oligodeoxynucleotides can impair production of VEGF in effector cells at the RNA level.[233] These compounds have the pharmacological advantage that they are small and highly specific. They may have the theoretical disadvantage of blocking both basal and induced VEGF expression. In the mouse model of retinopathy of prematurity, injection of these agents before the development of preretinal neovascularisation led to partial inhibition of VEGF production and preretinal angiogenesis.[233]

Recent work has shown that hypoxic induction of VEGF can be mediated by adenosine[166] and the adenosine A(2a) receptor,[168] and this may lead to therapeutic strategies aimed to specifically inhibit hypoxia induced VEGF production. However, this approach may be complex, because the action of adenosine proceeds via cyclic AMP, and cAMP is also a physiological mediator counteracting increased endothelial permeability.[248]

Neutralisation of free VEGF

Free VEGF can be neutralised by specific antibodies.[159][199][249] Intravitreal injections with such antibodies were able to prevent iris vascular changes in the monkey model of retinal ischaemia.[199] Alternatively, soluble VEGF receptor chimeric proteins may inhibit VEGF, as was shown to be effective in the mouse model of ROP.[232]

VEGF receptors and intracellular signal transduction pathways

Another approach in VEGF inhibition is aimed at blockage of the VEGF tyrosine kinase receptors or their intracellular transduction pathways.[163][164][250][251] In several experimental tumour models, transfection with a dominant negative VEGF receptor Flk-1 mutant was able to suppress tumour growth.[163][164]

The mitogenic effect of VEGF on endothelial cells appears to be mediated by certain isoforms of protein kinase C (PKC),[252][253] and specific PKC inhibitors are able to inhibit VEGF induced endothelial proliferation and angiogenesis.[250–253] However, such inhibitors are not specific for the eye, and much has to be learned about how they may affect wound healing and collateral formation.

Endothelial cellular response to VEGF

As outlined before, VEGF induces several functional changes in endothelial cells related to microvascular permeability or angiogenesis. These cellular response to the cytokine (and other angiogenesis factors) may be specifically inhibited—for example, by interfering with endothelial migration through integrin antagonists[73–75][254] or inhibitors of cell associated plasminogen activation.[72]

POTENTIAL SIDE EFFECTS OF VEGF INHIBITION

A matter of concern in the clinical use of VEGF inhibition is the possible role of the growth factor in physiological angiogenesis and other vital functions. Wound healing, the female reproductive cycle, collateral formation in cardiac ischaemia and other tissue responses may be influenced by interfering with VEGF activity (see earlier). Furthermore, the constitutive expression of the molecule in normal quiescent ocular[111][203][243][244] and other tissues[106][131–133][135] suggests unknown but potentially important functions. For example, basal production of VEGF by epithelia may maintain certain capillary beds, as has been suggested for the choroid plexus and the kidney.[106][134][135] Retinal pigment epithelium (RPE) in culture has been shown invariably to produce VEGF.[101][167][240][245] If a similar mechanism is operative between the RPE and the choroidal capillaries,[240][245] VEGF inhibition could lead to choroidal atrophy. Therefore, both systemic and local ocular inhibition of VEGF may have serious side effects.

PHARMALOGICAL ASPECTS AND DRUG DELIVERY OF VEGF INHIBITORY AGENTS

Application of medical inhibition of ocular angiogenesis or macular oedema will present ophthalmologists with many new problems in practical clinical management. Certain conditions may require short term or long term application, possibly through new drug delivery devices. Recently, several new drug delivery systems have been developed.[255–257] This technology could help to target stable VEGF inhibitors to the posterior segment. For less stable protein agents, gene therapy aimed at temporary expression of these compounds by the retinal pigment epithelium may prove useful in the future.

Conclusion

VEGF appears to play an important role as a vascular permeability and angiogenesis factor in the developing eye and in pathological eye conditions. It is amazing that the predictions of investigators like Michaelson and Ashton have been confirmed after almost 50 years. In the past few years our understanding of the molecular mechanisms of ocular angiogenesis has dramatically increased. More important, inhibitors of this growth factor may provide clinicians with powerful tools to treat the common ocular diseases in which angiogenesis or loss of the blood-retinal barrier cause visual impairment.

However, it should be realised that there is still much to be learnt about the exact role of VEGF and other angiogenesis factors, and their interactions, in pathological conditions as well as in the normal eye.[11] As with other growth factors, the actions of VEGF seem to be contextual, depending upon expression of receptors, the extracellular matrix, extravasated plasma proteins, other

cytokines and other, maybe unknown, influences.[12][44] Furthermore, the experimental animal models of ocular angiogenesis, from which we have learned most of the role of VEGF in the eye, are quite different from the human disease states, most notably in their time course and pathogenesis.

However, when all the experimental findings are taken together, VEGF has emerged as a major angiogenesis factor in the developing and adult human eye. Modulation of VEGF expression and its effect on angiogenesis and vascular permeability will give us new insights in pathological mechanisms at play in the eye. It is through these insights and by learning the interactions of VEGF with other mediators of angiogenesis that we can hope to develop new means of preventing or treating conditions which all too often lead to significant visual loss or blindness.

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