

Gene therapy for ocular angiogenesis

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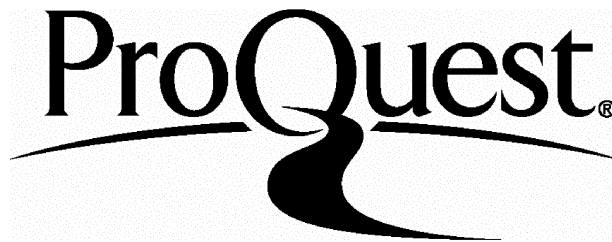
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abstract

This thesis describes a programme of work to develop local gene transfer of angiostatic proteins for the treatment of retinal and choroidal neovascular disorders. Current treatments are of limited efficacy and associated with significant adverse effects. Characterisation of the molecular and cellular events involved in angiogenesis has led to the identification of a number of angiostatic molecules with potential therapeutic value. The systemic administration of small molecule angiostatic proteins risks significant systemic adverse effects and if delivered intraocularly their half-life is short. Local gene transfer, however, offers the possibility of targeted, sustained and regulatable expression of angiostatic proteins to the retina after a single procedure to introduce a vector to an intraocular site. In order to investigate the potential value of this approach relevant animal models of retinal and choroidal angiogenesis were established and optimised. Viral vectors including adenoviral, adeno-associated viral and lentiviral vectors, expressing reporter genes and angiostatic proteins were produced. Reporter gene studies were performed to determine the kinetics of transgene expression following intraocular vector delivery in the models. Gene delivery of a vascular endothelial growth factor (VEGF) inhibitor, soluble Flt-1, significantly reduced neovascularisation in models of both retinal and choroidal angiogenesis, and had no apparent adverse effect in normal animals. Targeting of transgene expression to sites and periods of active neovascularisation was achieved by the incorporation of a hypoxia-responsive promoter into the gene construct. Pre-clinical studies of reporter gene transfer in a large animal model following subretinal delivery of a recombinant adeno-associated viral vector demonstrated efficient sustained reporter gene expression in cells of the outer retina. The findings of these studies were used to develop a proposal for a clinical phase I/II trial of gene therapy for choroidal neovascularisation in age-related macular degeneration by adeno-associated virus-mediated local expression of the VEGF inhibitor soluble sFlt-1.

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abbreviations

AAV	adeno-associated virus
Ad	adenovirus
AGE	advanced glycation end products
AMD	age-related macular degeneration
CMV	cytomegalovirus
cPPT	central polypurine tract
ECM	extracellular matrix
ERG	electroretinogram
eNOS	endothelial nitric oxide synthase
FGF	fibroblast growth factor
FIV	feline immunodeficiency virus
GH	growth hormone
GFP	green fluorescent protein
GTAC	Gene Therapy Advisory Committee
HIF-1	hypoxia-inducible factor-1
HIV	human immunodeficiency virus
HRE	hypoxia-response element
ICAM-1	intercellular adhesion molecule-1
IGF-1	insulin-like growth factor-1
iNOS	inducible nitric oxide synthase
MCP-1	monocyte chemoattractant protein-1
MMP	matrix metalloproteinase
NO	nitric oxide
NOS	nitric oxide synthase
NP-1	neuropilin-1
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEDF	pigment epithelium-derived factor
PKC	protein kinase C

RPE	retinal pigment epithelium
SDT	spontaneously diabetic Torii
SIV	simian immunodeficiency virus
TGF-beta	transforming growth factor-beta
TIMP	tissue inhibitor of metalloproteinases
WPRE	woodchuck post-transcriptional regulatory element
ZFP	zinc finger protein

publications

Work presented in this thesis has contributed to the following publications.

1. **JWB Bainbridge**, C Stephens, K Fowler, C Demaison, A Halfyard, AJ Thrasher and RR Ali. *In-vivo* gene transfer to the mouse eye using an HIV-based lentiviral vector: efficient long-term transduction of corneal endothelium and retinal pigment epithelium. *Gene Therapy* 2001;8:1665-1668
2. MB Reichel, **JWB Bainbridge**, D Baker, AJ Thrasher, SS Bhattacharya, RR Ali. An immune response after intraocular administration of an adenoviral vector containing a beta galactosidase reporter gene slows retinal degeneration in the rd mouse. *Br J Ophthalmol* 2001;85: 341-344
3. GM Sarra, C Stephens, F Schlichtenbrede, **JWB Bainbridge**, AJ Thrasher, PJ Luthert, RR Ali. Kinetics of transgene expression in mouse retina following subretinal injection of recombinant adeno-associated virus. *Vision Research* 2002;42: 541-549
4. **JWB Bainbridge**, A Mistry, M De Alwis, E Paleog, A Baker, AJ Thrasher and RR Ali. Inhibition of retinal neovascularisation by gene transfer of soluble VEGF receptor sFlt-1. *Gene Therapy* 2002;9: 320-326
5. **JWB Bainbridge**, H Jia, D Selwood, RR Ali, and I Zachary. Peptides Encoded by exon 6 of VEGF inhibit VEGF-induced angiogenesis in vitro and ischaemic retinal neovascularisation in vivo. *Biochem Biophys Res Commun* 2003; 302(4):793-9
6. **JWB Bainbridge**, A Mistry, K Binley, M De Alwis, A Thrasher, S Naylor, R Ali. Hypoxia-regulated transgene expression in experimental retinal and choroidal neovascularisation *Gene Therapy* 2003;10: 1049-54
7. **JWB Bainbridge**, A Mistry, FC Schlichtenbrede, A Smith, C Broderick, M De Alwis, A Georgiadis, PM Taylor, M Squires, C Sethi, D Charteris, AJ Thrasher, D Sargan and RR Ali. Stable rAAV-mediated transduction of rod and cone photoreceptors in the canine retina. *Gene Therapy -in press*
8. **JWB Bainbridge** A Mistry, A Thrasher, RR Ali. Gene therapy for ocular angiogenesis. *Clinical Science -in press*

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Much of the work presented is the result of collaborations. Ewa Paleog and Andrew Baker were involved with the adenoviral studies, Katie Binley and Stuart Naylor with the hypoxia-response element, and Ian Zachary with the VEGF peptides. David Sargan and Polly Taylor were involved in the canine studies, and David Charteris and Charanjit Sethi contributed data on retinal gene transfer in cats.

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1

Introduction

1.1 Clinical significance of ocular angiogenesis

Angiogenesis is the formation of new vasculature by the out-sprouting of vascular endothelial cells from existing vessels. This process is critical for embryonic development, growth, endometrial and placental proliferation, wound healing, and revascularisation of ischaemic tissues. Angiogenesis is also a central feature of many important diseases including cancer, rheumatoid arthritis, atherosclerosis and ocular neovascularisation. Pathological angiogenesis occurs in retinopathy of prematurity (ROP), proliferative diabetic retinopathy and age-related macular degeneration which are the leading causes of blindness in infants, individuals of working age and the elderly respectively.¹⁻³ Neovascularisation in these conditions causes visual loss through increased vascular permeability leading to retinal oedema, vascular fragility resulting in haemorrhage, or fibro-vascular proliferation with tractional and rhegmatogenous retinal detachment. Pathological angiogenesis occurring at other intraocular sites including the cornea and iris can also result in loss of vision through loss of corneal transparency and glaucoma respectively (Figure 1.1). Although neovascularisation tends to occur at a relatively late stage in the course of many ocular disorders it is nonetheless a highly attractive target for therapeutic intervention since it represents a final common pathway in processes that are multifactorial in aetiology and is the event that typically leads directly to visual loss.

1.1.1 Proliferative diabetic retinopathy

Diabetic retinopathy is the commonest cause of visual impairment in people of working age.² In this condition hyperglycaemia results in retinal microvascular

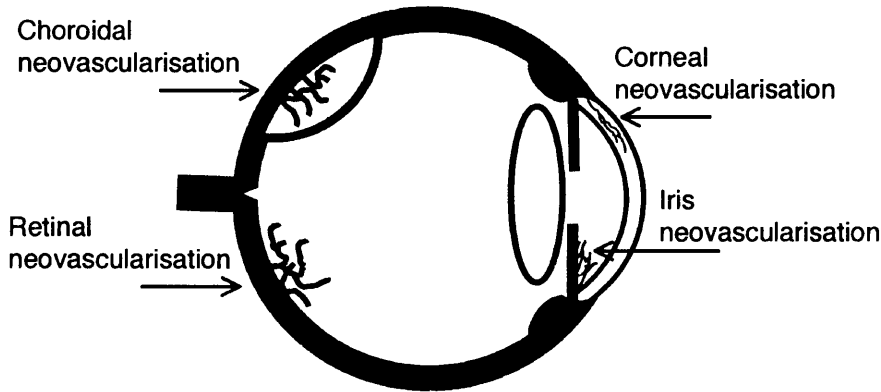


Figure 1.1 Schematic section of the eye showing common sites of intraocular neovascularisation

Retinal neovascularisation is typically seen in ischaemic retinopathies and extends pre-retinally into the vitreous cavity. Neovascularisation of the choroid is most commonly due to age-related macular degeneration and often extends subretinally.

occlusion and ischaemia. The subsequent hypoxia-induced upregulation of angiogenic growth factors results in neovascularisation that extends from the inner retinal surface into the vitreous gel (Figure 1.1).⁴ Complications of neovascular proliferation are the major causes of persistent severe visual loss in diabetes through haemorrhage into the vitreous (Figure 1.2b) or associated tractional retinal detachment.⁵ Although such complications of diabetes may be prevented by rigorous control of blood glucose^{6,7} and blood pressure⁸ this can be difficult to achieve in practice. The current conventional treatment for established proliferative diabetic retinopathy is pan-retinal laser photocoagulation.⁹ This technique is able to induce regression of retinal neovascularisation if initiated promptly, but is inherently destructive, and is itself associated with significant predictable adverse effects on visual function.¹⁰ Advanced proliferative diabetic retinopathy with persistent vitreous haemorrhage or associated tractional retinal detachment typically requires surgical intervention by vitrectomy.¹¹

Although ischaemia-induced retinal neovascularisation is most frequently seen in diabetic eye disease, it is also a central feature of other common retinopathies including retinopathy of prematurity, retinal vascular occlusion, sickle cell disease, carotid artery disease and ocular inflammatory disorders.

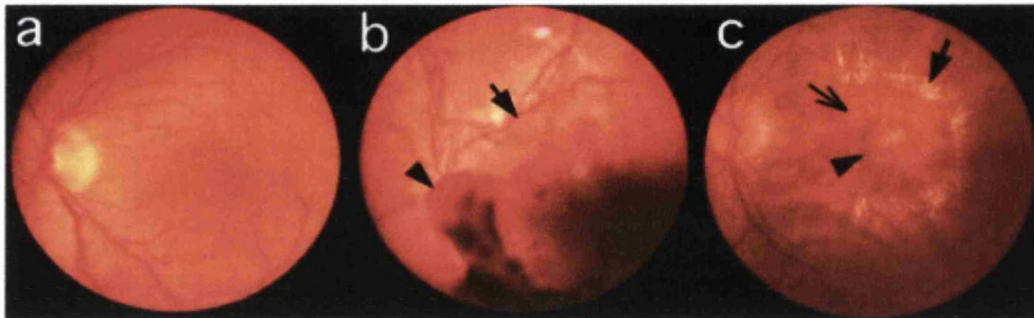


Figure 1.2 Fundus photographs showing retinal and choroidal neovascularisation

a, Normal fundus; b, Retinal neovascularisation in diabetic retinopathy. Fine new vessels are visible along the superotemporal vascular arcade (arrow) and vitreous haemorrhage, presumably originating from new vessels at the optic disc (arrowhead) extends inferiorly; c, Choroidal neovascularisation in age-related macular degeneration. Drusen (open arrow) and a ring of lipid exudates (arrow) are evident at the macula. A subtle grey area centrally (arrowhead) is evidence of the presence of a choroidal neovascular complex.

1.1.2 Neovascular age-related macular degeneration

Age-related macular degeneration (AMD) is the most common cause of blindness in industrialized countries, with an estimated incidence of 20 000 new cases annually in the UK and prevalence of 1.9% in people older than 50 years.¹² The pathogenesis of AMD is not well understood but involves abnormalities of the extracellular matrix at the level of Bruch's basement membrane.¹³ Choroidal neovascularisation in this context may be the result of hypoxia/ischaemia of overlying retinal pigment epithelial (RPE) cells, due either to the thickening of Bruch's membrane or to abnormalities of

choroidal perfusion,¹⁴⁻¹⁶ leading to the expression of pro-angiogenic cytokines.^{17,18}

Exudation or haemorrhage from resulting neovascular complexes (Figure 1, Figure 2) that extend from the choroidal vasculature through breaks in Bruch's membrane account for 80% of severe visual loss in this condition.^{19,20}

The established treatment modalities for choroidal neovascularisation in AMD comprise thermal laser photocoagulation and photodynamic therapy. These ablative approaches can offer short-term benefit to certain subgroups of patients²¹⁻²³ but are associated with significant adverse effects.^{10,24} Laser photocoagulation can be beneficial for the small minority of patients who have well-demarcated ("classic") neovascular lesions that do not extend beneath the fovea.^{24 25} Photocoagulation destroys photoreceptor cells, however, creating a central scotoma such that treatment of subfoveal lesions results in destruction of the foveal photoreceptors with permanent loss of central vision.²⁴ Photodynamic therapy is a non-thermal process that aims to selectively destroy new blood vessels by irradiation following the intravascular administration of a photosensitising agent. Since this approach does not result in the destruction of overlying photoreceptor cells it can be effective in patients with subfoveal neovascularisation and delays loss of vision in a subgroup of patients with predominantly classic subfoveal lesions. Both photocoagulation and photodynamic therapy are designed to eliminate abnormal blood vessels, but since these treatments fail to address the underlying stimuli for blood vessel growth they are associated with high rates of persistent and recurrent disease. Following photocoagulation the cumulative proportion of treated eyes with recurrent/persistent choroidal neovascularisation is approximately 50% after 3 years²⁶ and is accompanied by an increased frequency of severe visual loss.²⁷ Following photodynamic therapy, persistence and recurrence of neovascularisation are also common and frequently require further treatments.²²

Alternative experimental ablative approaches to choroidal neovascularisation include external beam radiotherapy and transpupillary thermotherapy. External beam radiation is of limited efficacy²⁸ and does not completely destroy CNV so there is a high risk of

re-activation or regrowth.²⁹ Transpupillary thermotherapy has been observed to cause closure of choroidal neovascular membranes^{30,31} but has not been shown to confer a benefit to vision in a controlled trial.

The surgical excision of subfoveal choroidal neovascularisation may stabilize or improve visual acuity in selected cases.²⁹ Visual prognosis is most dependent on the integrity of the subfoveal RPE-Bruch's-choriocapillaris complex after removal of the CNV.³² RPE incorporation into subfoveal membranes remains a limiting factor in AMD because surgical excision is typically complicated by concurrent removal of RPE.³³ RPE patch autotransplants³⁴ and human fetal RPE allografts³⁵ offer the potential means to repopulate the subretinal space but do not directly address the problems of CNV recurrence following surgical excision.³⁶ Excision of CNV in disorders other than AMD is more encouraging,³⁷ at least in part because the RPE Bruch's-choriocapillaris complex is often preserved after surgery in these conditions. Excision of subfoveal CNV in punctate inner choroidopathy³⁸ and the presumed ocular histoplasmosis syndrome³⁹ has been promising. Even in these conditions, however, CNV recurrence is a major limiting factor, occurring in up to 44 % of patients.^{40,41} Experimental surgical procedures are being developed to reposition the neurosensory fovea away from abnormal RPE and/or choroid to a new location that has presumably healthier retinal RPE and choriocapillaris in order to recover or preserve foveal function. Translocation of the fovea away from such subfoveal choroidal neovascularisation also permits the removal of the choroidal neovascularisation or its destruction by laser photocoagulation with minimal risk to the fovea.⁴² Recurrent neovascularisation is common, however, following such procedures, often occurs at the new fovea and is an important cause of vision loss.⁴³

1.1.2 Angiogenesis elsewhere in the eye

Pathological angiogenesis also occurs in tissues in the anterior segment of the eye (Figures 1.1 and 1.3). Neovascularisation of the iris typically occurs in the context of ischaemic retinopathies including central retinal vein occlusion and diabetes and can

cause loss of vision through associated closure of the irido-corneal drainage angle resulting in raised intraocular pressure and glaucomatous optic neuropathy.

Neovascularisation of the cornea can occur in response to a number of different insults including trauma, infection, inflammation and contact lens wear and results in loss of normal corneal transparency.⁴⁴ This project, however, is directed towards neovascular disorders of the retina and choroid since it is these conditions that are responsible for the major proportion of visual loss due to angiogenesis.⁴⁴

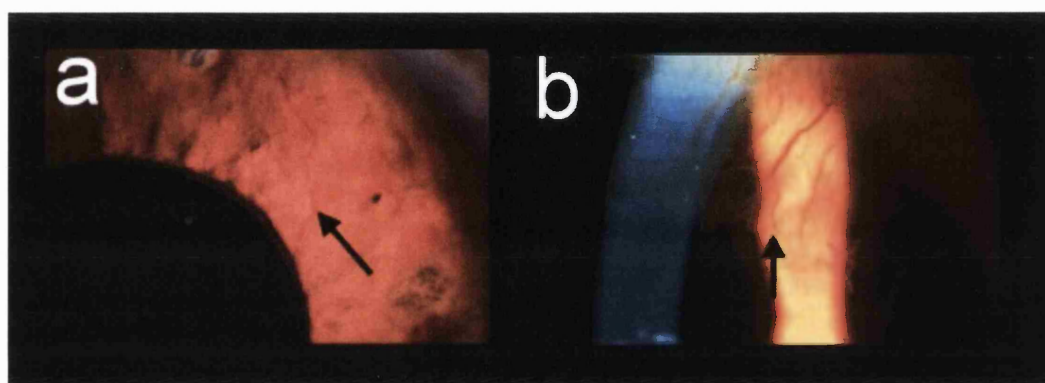


Figure 1.3 Anterior segment photographs showing neovascularisation of iris and cornea

a, Iris neovascularisation; b, Corneal neovascularisation

1.2 Molecular biology of ocular angiogenesis; potential targets for angiostatic therapies

Since available treatments for ocular neovascular disorders are limited, there is a clear clinical need for the development of novel approaches that are directed against the underlying pro-angiogenic stimuli so as to achieve a sustained therapeutic effect. The development of such treatments depends on a clear understanding of the molecular and cellular processes involved in angiogenesis.

Early studies of neovascularisation in the retina contributed significantly to the understanding of the pathogenesis of angiogenesis. In 1948 Michaelson investigated retinal vascular development and pathological retinal neovascularisation using perfusion techniques in human and cat fetuses.⁴⁵ He showed that the formation of retinal capillaries was predominantly a function of the retinal veins and described the capillary free zone that existed around arteries both in the immature and in the mature retina. It was on the basis of these observations that he formed the hypothesis that there existed in the retina a factor capable of affecting the growth of new blood vessels both in development and in disease. He argued that this factor, possibly of a biochemical nature, is present in the extra-vascular tissue of the retina in a concentration gradient that determined the extent of capillary growth from veins in development and in disease, and suggested that the stimulus for new vessel growth was associated with the metabolic demand of the retinal tissue.

In his Proctor Award Lecture of 1957,⁴⁶ Norman Ashton described how, though the nature of the stimulus for retinal vessel growth was still unknown, his own recent laboratory studies of retrolental fibroplasia suggested that Michaelson's vasoformative factor arose specifically in areas of low oxygen tension. He described in the cat retina how the immature vasculature uniquely underwent vaso-obliteration as a result of exposure to hyperoxia and determined that this effect became irreversible after continuous exposure for a minimum period of time. Subsequent vaso-proliferation occurred in proportion to the degree of vaso-obliteration and arose from those vessels immediately adjacent to the obliterated areas. He concluded that the factor which stimulates growth of vessels in the retina is formed in hypoxic tissue and speculated that it might derive from the products of anaerobic metabolism. During the same period, Wise proposed that in all proliferative retinopathies, retinal capillary or vein obstruction resulted in the production of a vasoproliferative stimulus by hypoxic tissue leading to the development of neovascularisation.⁴⁷ Following these early observations this mechanism of ischaemia-induced upregulation of angiogenic growth factors is now well-established as a central to the pathogenesis of retinal neovascularisation⁴⁸ and

has also been implicated in the development of choroidal neovascularisation.¹⁸ The cytokine vascular endothelial growth factor (VEGF) has been identified as a major candidate for Michaelson's vasoformative factor though it appears that angiogenesis is regulated not by a single factor but by a balance between local stimulators including VEGF and inhibitors such as pigment epithelium-derived factor (PEDF).⁴⁹

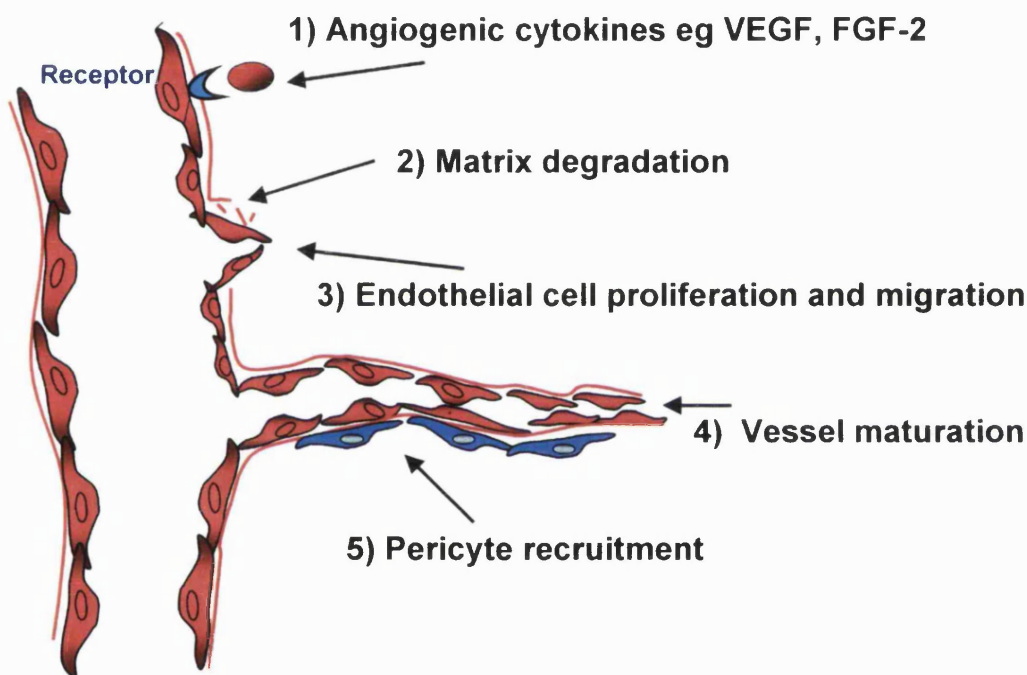


Figure 1.4 Schematic diagram illustrating stages of angiogenesis

Angiogenesis is a complex multi-step process that involves the out-sprouting of vascular endothelial cells from existing vessels through endothelial cell proliferation and migration, extracellular matrix remodelling and capillary tube formation (Figure 1.4). This process is controlled by complex interactions between growth factors, extracellular matrix and cellular components, the net outcome being determined by the balance of angiogenic and angiostatic elements.⁵⁰ A number of growth factor molecules are involved in the control of angiogenesis and the therapeutic manipulation of one or a combination of these offers the potential means to control neovascularisation in the

eye. Cytokines that have been effectively targeted in experimental models include vascular endothelial growth factor, insulin-like growth factor-1, pigment epithelium-derived factor, matrix metalloproteinases, angiostatin, endostatin, angiopoietin and integrins. Additional targets in diabetic retinopathy include aldose reductase, advanced glycation end products, protein kinase C and nuclear factor kappa B. Although there are at present no established molecular treatments for proliferative diabetic retinopathy or choroidal neovascularisation, a number of experimental molecular therapies are currently under evaluation in clinical trials (Table 1.1).

1.2.1 Vascular endothelial growth factor

VEGF is a potent endothelial cell-specific mitogen that plays a critical role in angiogenesis.^{51,52} A 46kDA homodimeric glycopeptide, VEGF exists in three isoforms in the mouse and four isoforms in the human as a result of alternative splicing. VEGF is expressed by several different ocular cell types including pigment epithelial cells, pericytes, vascular endothelial cells, neuroglia and ganglion cells,^{18,51,53,54} and in specific spatial and temporal patterns during retinal development.⁵⁵ Expression of VEGF is upregulated by hypoxia *in vitro*⁵⁶ and *in vivo*⁵⁴ through a hypoxia-inducible factor-1 (HIF-1) transcriptional element.⁵⁷ VEGF isoforms act via specific *fms*-like receptors, *Flt-1* and *Flk-1* /KDR, which are high-affinity receptor tyrosine kinases expressed on vascular endothelial cells that on binding VEGF phosphorylate a number of proteins including phospholipase C γ . This leads to the formation of diacylglycerol (DAG), activation of protein kinase C (PKC) and ultimately to endothelial cell proliferation, migration, and increased vasopermeability. Placental growth factor (PlGF) is a member of the VEGF family that stimulates endothelial proliferation *in vivo* and angiogenesis *in vitro* and appears to potentiate the angiogenic effect of VEGF either by enhancing its expression or by the formation of heterodimers.⁵⁸ A soluble truncated form of the VEGF receptor Flt-1, sFlt-1, is the only known endogenous specific inhibitor of VEGF.^{59,60} sFlt-1, which lacks the membrane-proximal immunoglobulin-like domain, the trans-membrane spanning region and the intracellular tyrosine-kinase domain, is generated by alternative splicing. Its inhibitory

activity results from sequestration of VEGF, to which it binds with high affinity, and from formation of inactive heterodimers with membrane-spanning isoforms of the VEGF receptors Flt-1 and KDR.^{59,61}

VEGF plays a central role in a number of angiogenic disorders including tumorigenesis⁶² and rheumatoid arthritis⁶³ in addition to retinal neovascular disease. VEGF levels are increased in experimental models of retinal ischaemia,^{53,64} in patients with proliferative diabetic retinopathy,⁶⁵⁻⁶⁷ retinopathy of prematurity,⁶⁸ retinal vein occlusion,⁶⁶ and in choroidal neovascularisation.⁶⁹ Injection of recombinant VEGF protein into the eyes of non-human primates produces neovascularisation of the iris.⁷⁰ and overexpression of VEGF by photoreceptors in transgenic mice causes neovascularization in the retina.⁷¹ Adenovirus-mediated VEGF overexpression in the outer retina causes neovascularisation in the subretinal space.⁷² Conversely, antagonists of VEGF inhibit experimental neovascularisation in the iris⁷³ retina,^{52,60,74} and choroid.⁷⁵

The central role of VEGF in angiogenesis makes it an attractive target molecule for angiostatic strategies both in the eye and elsewhere. The endogenously expressed soluble VEGF receptor, sFlt-1, has attracted particular attention for its potential therapeutic role in the control of neovascularisation.^{76,77} Other anti-VEGF strategies to control ocular neovascularisation in experimental models have included neutralising anti-VEGF monoclonal antibodies^{73,78}, soluble VEGF-receptor chimeric proteins,⁶⁰ oligonucleotides,^{74,79} and inhibition of a VEGF-specific protein kinase.⁵² Two experimental treatments directed at preventing choroidal neovascularisation in AMD through targeting of VEGF are currently under evaluation in clinical trials. EYE001 (macugen) is a VEGF pegylated RNA aptamer (an oligonucleotide that acts like a high-affinity anti-VEGF antibody)⁸⁰ and RhuFab V2 (AMD Fab) is a monoclonal antibody fragment directed against VEGF.⁸¹ Both these agents are administered by repeated intravitreal injection.

Table 1.1 Experimental molecular therapies for retinal and choroidal neovascularisation evaluated in clinical trials

Condition	Agent	Mechanism of action	Route of administration
Proliferative diabetic retinopathy	sorbinil ⁸²	aldose reductase inhibitor	oral dosing
	LY333531 ^{83,84}	beta-selective protein kinase C inhibitor	oral dosing
	octreotide ^{85,86}	somatostatin analogue; GH/IGF-1 inhibition	repeated subcutaneous injections
	pegvisomant ⁸⁷	Growth hormone receptor antagonist	repeated subcutaneous injections
Neovascular AMD	EYE001 (macugen) ⁸⁰	VEGF inhibition by pegylated RNA aptamer	repeated intravitreal injection
	RhuFab V2 (AMD Fab) ⁸¹	VEGF inhibition by monoclonal antibody	repeated intravitreal injection
	ADGVPEDF.11D ⁸⁸	PEDF overexpression	single intravitreal injection of adenoviral vector
	anecortave acetate	angiostatic steroid	single periocular injection
	triamcinolone acetonide	angiostatic steroid	intravitreal injection
	squalamine	angiostatic steroid	repeated intravenous injections
	AG3340 (Prinomastat) ⁸⁹	synthetic MMP inhibitor	oral dosing

1.2.2 Pigment epithelium-derived factor

Pigment epithelium-derived factor (PEDF) is a soluble angiostatic protein secreted by retinal pigment epithelial cells in the developing and adult retina. It is a non-inhibitory member of the serine protease inhibitor (serpin) superfamily of proteins⁹⁰ and was first described for its neurotrophic properties.⁹¹ PEDF induces neuronal differentiation of retinoblastoma cells⁹¹ and promotes neurite-outgrowth and survival of cerebellar granule neurons. The PEDF gene is closely linked to an autosomal-dominant locus for retinitis pigmentosa and it has been suggested that PEDF may act as a survival factor for photoreceptors.⁹² PEDF interacts with receptors present on photoreceptors⁹³ and protective effects against photoreceptor death in mouse models of inherited retinal degeneration⁹⁴ and after light damage⁹⁵ have been described. The angiostatic effect of PEDF may be the result of promotion of endothelial cells apoptosis.⁹⁶ PEDF expression is down-regulated in hypoxia and its loss appears to play a permissive role in ischemia-driven retinal neovascularisation.⁹⁷ The combination of angiostatic and neuro-protective properties of PEDF make this a potentially attractive candidate for the control of neovascularisation in age-related maculopathy since degeneration of the retina is also a typical feature of this condition. A clinical trial of PEDF for neovascular AMD is currently ongoing.⁸⁸

1.2.3 Growth hormone and insulin-like growth factor

The growth hormone (GH) - insulin-like growth factor-1 (IGF-1) axis is known to play a role in modulating retinal angiogenesis since hypophysectomy results in regression of diabetic retinopathy. IGF-1 mediates the mitogenic effect of GH. Ischaemia-induced retinal neovascularisation is reduced in transgenic mice expressing a GH antagonist gene and in normal mice given an inhibitor of GH secretion, and this effect is reversed by exogenous IGF-I administration.⁹⁸ IGF-1 induces angiogenesis experimentally⁹⁹ and its vitreous concentration is significantly raised in proliferative diabetic retinopathy.⁶⁷ IGF-1 appears to play a permissive role in the development of ischaemia-induced retinal neovascularisation and is able to induce retinal neovascularisation directly.⁹⁹

Both local and systemic expression of IGF-1 appear to contribute to its intraocular levels.¹⁰⁰ IGF-1 inhibition by a receptor antagonist suppresses experimental retinal neovascularisation.⁹⁸ Although a clinical trial of the growth hormone receptor antagonist pegvisomant has not demonstrated regression of diabetic retinopathy,⁸⁷ trials of GH/IGF-1 axis inhibition using somatostatin analogues suggest that this approach may offer a beneficial effect in diabetic retinopathy^{85,86} and might also be effective in choroidal neovascularisation.¹⁰¹

1.2.4 Transforming growth factor-beta

The transforming growth factor-beta (TGF-beta) superfamily is a large family of multifunctional factors that regulate various cellular functions, including cellular proliferation, migration, differentiation, apoptosis and extracellular matrix production. The mechanism by which TGF-beta stimulates angiogenesis is not well understood, but it appears to have an effect on the regulation of VEGF gene expression at the transcriptional level¹⁰² and may also act by recruiting inflammatory cells capable of stimulating angiogenesis directly.¹⁰³ TGF-beta 1 down-regulates vascular endothelial growth factor receptor 2 (FLK) expression in vascular endothelial cells.¹⁰⁴ TGF-beta is expressed in ocular tissues including the cornea, ciliary epithelium, lens epithelium, retina, and blood vessels and appears to play a role in choroidal neovascularisation, being upregulated in experimental models¹⁰⁵ and in human specimens.¹⁰⁶ Blockade of TGF-beta using a soluble TGF-beta receptor inhibits corneal angiogenesis.¹⁰⁷

1.2.5 Fibroblast growth factors

Fibroblast growth factors (FGFs) are a family of 9 heparin-binding polypeptides with mitogenic and neurotrophic properties. FGF-1 (acidic) and FGF-2 (basic) are well-described as modifiers of angiogenesis.¹⁰⁸ The other members of the FGF family are less well characterised but appear to be important mediators of development.¹⁰⁹ Many of the biological activities of the FGFs have been found to depend on their receptors'

intrinsic tyrosine kinase activity and second messengers such as the mitogen-activated protein kinases. Intracellular FGFs might also have a direct biological role within the nucleus; the absence of a signal sequence to direct their secretion and their ability to traffic to the nucleus are unique structural features that may be relevant to the regulation of their activities.¹¹⁰ Both FGF-1 and FGF-2 are involved in retinal development and angiogenesis¹¹¹ and appear to have neurotrophic effects on retinal cells.^{112,113} Although FGF-2 induces angiogenesis in the choroid^{114,116} it appears to be neither necessary nor sufficient alone for the development of retinal neovascularization.¹¹⁷

1.2.6 Platelet-derived growth factor

Platelet-derived growth factor (PDGF) is a 45 kDa single chain polypeptide that has migratory and proliferative effects on retinal glial and endothelial cells¹¹⁸ PDGF is upregulated during ischaemia -induced retinal neovascularisation¹¹⁹ and is also implicated in the induction of proliferative vitreoretinopathy, being upregulated both in experimental models¹²⁰ and in human disease.^{121,122}

1.2.7 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of zinc-binding, calcium ion-dependent, neural endopeptidases that function as proteolytic enzymes in the degradation processes of the extracellular matrix (ECM)¹²³ and have important roles in development, wound healing and angiogenesis. Their inactive precursors are activated locally by proteolytic removal of amino acid terminal ends and their activity is further regulated at the transcriptional level by cytokines. The tissue inhibitors of metalloproteinases (TIMPs) are a family of endogenous inhibitors that act to protect matrix by down regulating MMP activity. MMPs-1,-2 and -9 are believed to initiate and promote angiogenesis. MMP-2 expression by endothelial cells is upregulated by hypoxia and by VEGF¹²⁴ and interacts with alpha v beta 3 integrin on endothelial cell surface to create localised areas of high proteolytic activity. MMP-2 and MMP-9

preferentially degrade basement membrane components such as type IV collagen. Angiogenesis is disrupted by PEX, a non-catalytic C-terminal hemopexin domain of MMP-2 with integrin binding activity.^{125,126} MMPs-7,-9 and -12 may block angiogenesis by converting plasminogen to angiostatin which is one of the most potent angiogenesis antagonists.¹²⁷ TIMPs-1,-2,-3 and possibly TIMP-4 inhibit neovascularisation.¹²⁸⁻¹³⁰ Critical roles for MMPs and TIMPs are implicated in ocular angiogenic disorders. MMPs-2 and -9 are implicated in both retinal¹³¹ and choroidal¹³² neovascularisation. TIMP-3 is synthesised by RPE cells¹³³ and is present in Bruch's membrane and choroid in association with the extracellular matrix. Mutations in the gene encoding TIMP-3 have been described in Sorsby's fundus dystrophy, a maculopathy that is characterised by thickening of Bruch's basement membrane and choroidal neovascularisation. Matrix degradation is an attractive target for angiostatic therapy because it represents a critical step and final common pathway in angiogenesis. AG3340 (Prinomastat), an oral synthetic inhibitor of MMP-2, -9, -13 and -14, inhibited tumour growth and angiogenesis in a variety of preclinical models but was ineffective in clinical trials for choroidal neovascularisation in AMD.⁸⁹

1.2.8 Integrins

Integrins are non-covalently associated transmembrane glycoprotein heterodimer receptors that mediate bidirectional interactions between ECM proteins and cytoskeleton across the plasma membrane and also function as signal transducers.¹³⁴ The integrins alpha v beta 3 and alpha v beta 5 are implicated in angiogenesis.¹³⁵ Both are expressed in neovascular endothelial cells in proliferative diabetic retinopathy whereas only alpha v beta 3 is expressed in active choroidal neovascularisation. Peptide antagonists administered systemically,¹³⁶ locally¹³⁷ or by topical application¹³⁸ result in inhibition of experimental retinal angiogenesis but these have yet to be evaluated in clinical trials.¹³⁹

1.2.9 Urokinase plasminogen activator

The urokinase plasminogen activator (uPA) system facilitates the movement of cells through a matrix, including migration of endothelial cells during angiogenesis, by initiating an extracellular cascade of proteolysis that involves the activation of plasminogen and MMPs.^{140,141} uPA is a serine protease secreted by vascular cells as a single chain inactive form that is proteolytically converted to an active form that modulates angiogenesis by remodelling of extracellular matrix. In addition to dissolution of matrix, proteolytic cascades activate certain growth factors such as TGF-beta or K1-5¹⁴² and release others such as FGF-2 that are sequestered on the cell surface or within the ECM, thus contributing to the evolution of a migratory or invasive cell phenotype. uPA is also able to modulate endothelial cell adhesion, migration and proliferation responses in a non-proteolytic fashion through signalling involving its specific cell surface receptor uPAR. uPAR itself lacks intracellular signalling domains and such signals are believed to be transduced via interactions between uPA/uPAR and integrins.¹⁴³ The uPA system has been implicated in the development of human diabetic neovascular membranes.¹⁴⁴ A secreted version of the amino-terminal fragment (ATF) of murine uPA antagonizes uPA binding to uPAR. ATF inhibits cell invasion in vitro and angiogenesis in experimental tumours,¹⁴⁵ but this approach has yet to be applied to ocular neovascular disease.

1.2.10 Angiostatic steroids

Angiostatic steroids consist of glucocorticoids coupled to heparin. The mechanism of their angiostatic effect is believed to involve inhibition of uPA,¹⁴⁶ activation of MMPs and reduction in adhesion molecule expression¹⁴⁷ though inhibition of inflammatory cell-mediated expression of angiogenic factors may also be involved. Clinical trials of angiostatic steroids in neovascular AMD include periocular injection of anecortave acetate, that has a promising effect in experimental models,¹⁴⁶ and intravitreal injection of triamcinolone.^{148,149} Squalamine is a novel anti-angiogenic amino sterol that acts, at least in part, by blocking mitogen-induced proliferation and migration of endothelial

cells.¹⁵⁰ Administered intravenously, squalamine significantly improves intraocular neovascularisation in experimental models^{151,152} and a clinical trial of squalamine is currently underway to evaluate its effect in neovascular AMD.

1.2.11 Angiostatin and endostatin

Plasminogen is an inactive proenzyme that is converted by plasminogen activators to the active enzyme, plasmin, which is central to the fibrinolytic cascade. Angiostatin is a 38 kD internal fragment of plasminogen that comprises four kringle domains (kringle 1-4) and is an endogenous circulating inhibitor of angiogenesis first identified in mouse tumour models.¹⁵³⁻¹⁵⁵ Its anti-angiogenic effect appears to result from specific inhibition of endothelial cell proliferation,¹⁵⁶ and may involve an interaction with the integrin $\alpha(v)\beta(3)$ ¹⁵⁷ and increased endothelial cell apoptosis. The anti-proliferative activity of angiostatin on endothelial cells is shared by kringle 1, kringle 2, and kringle 3, but probably not by kringle 4, and its potency is increased when kringle 4 is removed.¹⁵⁸ Kringle 5 of plasminogen appears to be more potent than angiostatin *in vitro*,¹⁵⁹ and may act through down-regulation of VEGF and up-regulation of PEDF.¹⁶⁰ Intravitreal injection of Kringle 5 reduces experimental ischaemia-induced retinal neovascularisation *in vivo*.¹⁶¹

Endostatin is a 20 kDa C- terminal fragment of collagen XVIII released by elastase activity.¹⁶² Endostatin specifically inhibits endothelial proliferation *in vitro* and potently inhibits angiogenesis and tumor growth *in vivo*. Its mechanism of action is zinc-dependent and may involve blocking VEGF receptor signalling, interaction with integrins¹⁶³ or induction of endothelial cell apoptosis.¹⁶⁴ Both angiostatin and endostatin have yielded promising results in experimental models though these inhibitors have yet to be evaluated in clinical trials for ocular neovascular disorders.¹⁶⁵

1.2.12 Angiopoietins and Tie receptors

Tie receptors are endothelium specific receptor tyrosine kinases. The Tie1 receptor is required for the structural integrity of endothelial cells, but its ligand has yet to be identified. The Tie2 receptor has been implicated in stabilisation and maturation of vessels through the action of an agonist ligand, angiopoietin 1 (Ang1) and an antagonistic ligand, Ang2. Ang1 mediates vessel maturation and remodelling and is essential for normal vascular development in the mouse¹⁶⁶. It promotes survival of endothelial cells but not chemotaxis or proliferation and its effect appears to result from the protection of endothelial cells against apoptosis.¹⁶⁷ Ang1 also appears to confer resistance to VEGF-mediated vascular leakage.¹⁶⁸ Ang2 antagonises the Tie2 receptor and its expression is upregulated during physiologic and pathologic neovascularisation¹⁶⁹ sTie-2, a synthetic soluble receptor of angiopoietin inhibits angiogenesis and has potential therapeutic value. Ang1 is also of potential therapeutic value for reducing microvascular leakage in diseases in which the leakage results from chronic inflammation or elevated VEGF and, in combination with VEGF, for promoting the growth of non-leaky vessels.¹⁶⁸

1.2.13 Nitric oxide

The nitric oxide (NO) pathway plays an important role in physiological and pathological angiogenesis and offers a promising target for angiostatic therapeutic strategies. NO is a free radical that is produced from L-arginine by NO synthases (NOS)¹⁷⁰ and is involved in a range of diverse processes including neurotransmission, vascular permeability and inflammation. At least three different forms of NOS, each encoded by a specific gene, have been demonstrated.¹⁷⁰ Neuronal NOS (nNOS), present in neurons, and endothelial NOS (eNOS), predominantly found in vascular endothelial cells, are continuously expressed in the body whereas inducible NOS (iNOS) is expressed (in a range of cell types) only after transcriptional activation by endotoxins and cytokines.¹⁷¹ NO is known to influence neovascularisation in a variety of models of angiogenesis, and its effect can be either pro-angiogenic¹⁷² or anti-

angiogenic¹⁷³ depending on the different NOS isoform involved. There is evidence that eNOS plays a pro-angiogenic role¹⁷⁴ that involves the promotion of endothelial cell migration and differentiation,¹⁷⁵ possibly via the upregulation of alpha(v)beta(3) integrins.¹⁷⁶ Elevation of eNOS activity in correlation with angiogenesis has been extensively reported and eNOS inhibitors may offer a valuable angiostatic effect.¹⁷⁵ The role of iNOS in angiogenesis remains controversial. Although iNOS is expressed in the ischaemic retina¹⁷⁷ its effect can be anti-angiogenic.¹⁷⁸ iNOS may be responsible for misdirecting angiogenesis in ischaemic retinopathy away from the ischaemic retina and into the vitreous.¹⁷⁹ Inhibition of iNOS might then offer a very attractive opportunity to control of vitreal neovascularisation by facilitating appropriate re-vascularisation of hypoxic retina.

1.2.14 Pathogenesis of angiogenesis in diabetic retinopathy

In diabetic retinopathy the mechanisms by which hyperglycaemia leads to the upregulation of angiogenic growth factors (reviewed in detail elsewhere¹⁸⁰) are complex and involve activation of the polyol pathway, non-enzymatic glycation and oxidative stress; retinal hypoxia is the result of subsequent haemodynamic changes, microvascular occlusion and vascular cell apoptosis (Figure 1.5). This pathway offers a number of potential opportunities for specific molecular therapeutic intervention.

Hyperglycaemia activates the polyol pathway in which glucose is converted to sorbitol by endothelial aldose reductase leading to capillary cell death. Although inhibition of aldose reductase reduces the high glucose-induced death of retinal capillary cells *in vitro*,¹⁸¹ the effect of aldose reductase inhibitors in clinical trials of diabetic retinopathy is inconclusive.⁸² Hyperglycemia also leads to the irreversible formation of advanced glycation end products (AGEs) which in turn generate oxygen-derived free radicals that contribute towards increased oxidative stress and themselves increase autocrine retinal VEGF expression,¹⁸² and inhibition of AGE formation is another potential therapeutic strategy to target angiogenesis.^{183,184} Activation of endothelial protein kinase C (PKC) by diacylglycerol, synthesised either *de novo* in chronic hyperglycaemia or via VEGF receptor activation, results in the upregulation of

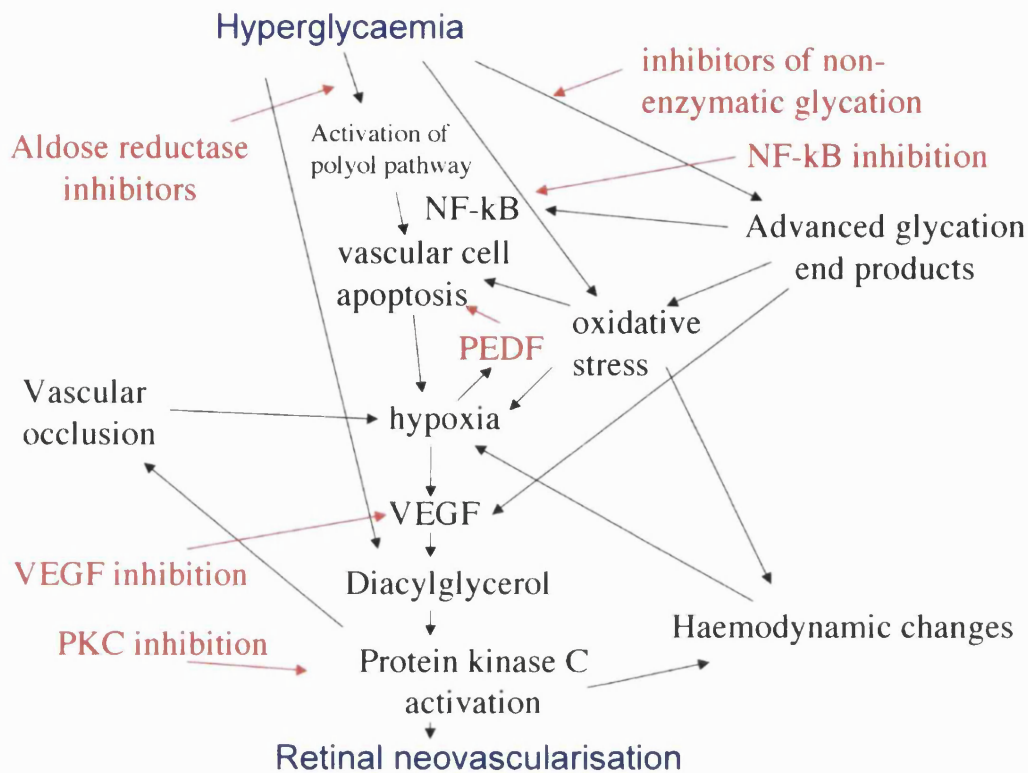


Figure 1.5 Schematic diagram illustrating major pathogenic pathways in proliferative diabetic retinopathy

Potential strategies for molecular therapeutic intervention are indicated (red arrows).

platelet-activating factor expression with leukocyte activation, thrombus formation, haemodynamic changes, and increased vascular cell permeability and proliferation. Interventions that increase diacylglycerol metabolism or inhibit PKC isozymes ameliorate the functional consequences of DAG-PKC activation in experimental models of diabetes¹⁸⁵ and reduce the extent of ischaemia-induced experimental ocular neovascularisation.¹⁸⁶ Clinical trials to evaluate the effect of the PKC inhibitor LY333531 in diabetic retinopathy are in progress.^{83,84} Death of retinal capillary cells can occur by apoptosis especially in the context of fluctuating glucose levels; AGEs may induce activation of nuclear factor kappa B (NF-kB), a molecule that appears to be an important signaling molecule in retinal vascular cell survival.^{184,187} Inhibition of

NFkB activation offers a potential strategy to prevent retinal hypoxia through the inhibition of vascular cell apoptosis.¹⁸⁷

Retinal neovascularisation may occur as a result of ischaemia in conditions other than diabetes, for example retinal vascular occlusion and carotid stenosis. In retinal vein occlusion neovascularisation appears to occur through hypoxia-induced upregulation of VEGF expression.¹⁸⁸ Tissue hypoxia appears to be a key step in triggering neovascularisation in experimental retinal vein occlusion,¹⁸⁹ and retinal VEGF expression is upregulated in both experimental vein occlusion in pigs¹⁹⁰ and in ischaemic retinal vein occlusion in patients.⁶⁶

Retinal neovascularisation may also be a feature of inflammatory eye disease. This may result from associated vascular occlusion leading to ischaemia and hypoxia-induced VEGF upregulation. This mechanism is clearly involved in occlusive vasculitides such as Bechet's disease and may also be the major mechanism in other conditions such as sarcoid uveitis.¹⁹¹ Alternatively, certain inflammatory cytokines are believed to have a direct angiogenic effect, and retinal neovascularisation may develop in patients with chronic uveitis in the absence of retinal ischaemia. In this situation regression of new vessels may be induced by immuno-suppression alone.¹⁹²

1.2.15 Pathogenesis of angiogenesis in age-related macular degeneration

Choroidal neovascularisation in AMD appears to be the combined result of local upregulation of angiogenic cytokines in association with an underlying abnormality of the extracellular matrix between RPE cells and the choroid. Though choroidal neovascularisation can be induced by laser rupture of Bruch's basement membrane, it is not induced by local upregulation of angiogenic factors in the absence of injury to Bruch's membrane.⁷¹ Abnormalities of the extracellular matrix are clinically evident in AMD as pale subretinal deposits known as drusen, and discontinuities in Bruch's basement membrane are a common feature of other conditions that are associated with

choroidal neovascularisation including high myopia, pseudoxanthoma elasticum and Sorsby's fundus dystrophy.¹⁹³ Patients with Sorsby's fundus dystrophy have mutations in the gene encoding TIMP-3, a protein that is involved in regulation of extracellular matrix turnover. Extracellular matrix deposition along Bruch's membrane and increased production of FGF-2 in RPE cells can both be caused by oxidative stress.¹⁹⁴ Choroidal neovascularisation may be the result of upregulated expression of pro-angiogenic cytokines, at least in part, in response to hypoxia/ischaemia of overlying retinal pigment epithelial cells,^{17,18} due either to thickening of Bruch's membrane or to abnormalities of choroidal perfusion,¹⁴⁻¹⁶

1.3 Strategies for molecular angiostatic therapies

It is clear that angiogenesis is a highly complex process and that the effects of cytokines on endothelial cell-matrix interactions can be contextual.¹⁹⁵ However, since determination of the angiogenic phenotype appears to be the result of a net balance of positive and negative regulators of blood vessel growth,¹⁹⁶ the introduction of a single agent to tip the balance towards angiostasis may be all that is required to achieve a valuable therapeutic effect. Effective intervention may be possible by targeting pathogenic processes at a number of levels in the relevant pathways.

As synergistic effects of angiostatic proteins have been described both *in vitro* and *in vivo*,¹⁹⁷ a combination of therapeutic factors, targeting independent pathways or different levels in the same pathway, may result in a particularly powerful effect. In both retinal and choroidal neovascular disorders, angiogenic cytokines such as VEGF are attractive targets and may be targeted directly or their pathways interrupted downstream, for example by specific protein kinase inhibitors. Alternative upstream targets tend to be disease-specific and might, for example, include AGEs in diabetic retinopathy or extracellular matrix deposition in AMD. In view of the complexity of these pathways, one might expect targeting upstream processes to be powerful though less specific, and conversely, downstream approaches to be relatively specific but less powerful. However, the possibility that compensatory mechanisms by alternative

pathways might neutralise the effect of intervention is difficult to predict and ultimately the optimal approach may be determined only by appropriate evaluation in experimental models and clinical trials.

1.4 Rationale for ocular gene transfer of angiostatic proteins

Though potentially efficacious, the systemic administration of angiostatic molecules risks important adverse systemic effects. In addition to its critical role in embryogenesis, physiological angiogenesis is central to wound healing and recovery from ischaemic events through revascularisation and the formation of collateral circulations. Patients with retinal neovascular disease, typically associated with diabetes or advanced age, are also likely to be at increased risk of ischaemic heart disease, cerebrovascular and peripheral vascular disease. The systemic inhibition of angiogenesis in these individuals would risk compromising critical vascular responses to ischaemic events. Clinical trials of VEGF antagonists for tumour therapy suggest that their systemic administration may also be associated with vascular toxicities such as haemorrhage and thrombo-embolic events.¹⁹⁸ In addition to these concerns, the production of recombinant proteins is technically difficult and their manufacture is expensive.¹⁶⁵ Since the access of proteins to the retina is restricted by the blood-retinal barrier, the high systemic doses required to achieve therapeutic intraocular levels would be particularly expensive and hazardous.

For these reasons, the local delivery of angiostatic agents offers significant potential advantages. Intraocular neovascularisation in experimental models is reduced by the repeated intravitreal injections of neutralizing anti-VEGF monoclonal antibodies,⁷³ recombinant soluble VEGF-receptor chimeric proteins⁶⁰ and antisense oligodeoxynucleotides.⁷⁴ The effective control of angiogenesis in patients with retinal neovascular disorders, however, is likely to require the sustained presence of the

angiostatic protein in the eye. The relatively short half-life of proteins delivered by intravitreal injection is such that frequently repeated administration would be necessary to maintain therapeutic levels¹⁹⁹ and would pose a high cumulative risk of local complications including intraocular infection, vitreous haemorrhage and retinal detachment.

In contrast, somatic gene transfer offers the possibility of localised, targeted, sustained and regulatable delivery of therapeutic proteins following a single procedure to introduce a vector to an appropriate intraocular site. Using a gene transfer approach, sustained expression can be achieved locally while minimising any risk of systemic adverse effects. Tissues may be targeted by vector design and surgical techniques, and expression may potentially be regulated through the use of tissue-specific, inducible or tissue-responsive promoters.

1.4.1 Vectors for ocular gene transfer

The eye is an ideal organ for *in-vivo* gene transfer. Ocular anatomy lends itself to the accurate delivery of vector suspensions because the tissues are compartmentalised and readily accessible by microsurgical techniques under direct visualisation. Its small size means that only tiny volumes of vector suspensions are required to transduce a significant proportion of cells in the target tissue, and using appropriate vectors, even non-dividing cell populations may be efficiently transduced by a single dose. The relative immune privilege of ocular tissues may confer some advantage in terms of long term transgene expression.²⁰⁰ The optical transparency of the eye enables GFP reporter gene expression to be observed *in-vivo* in many instances, and therapeutic effects on structure and function may be readily observed and quantified using a variety of techniques both experimentally and clinically.

A number of different viral and non-viral vector systems for gene transfer to ocular tissues have been extensively evaluated.^{201,202} Recombinant adenovirus (Ad), adeno-

associated virus (AAV), and lentivirus systems are the most frequently used viral vectors. The ideal choice of vector for a given ocular application is dependent on the natural tropisms of the vectors and their time-courses of expression.

1.4.1.1 Recombinant adenoviral vectors

Adenoviruses are a family of human DNA viruses that cause respiratory tract infections. Viral particles are 80-90 nm in diameter and are able to infect both dividing cells and those that are quiescent or terminally differentiated. First generation vectors are deleted in the E1 region, making them replication defective, and in the E3 region to accommodate DNA inserts of up to 7.5 kb. Ad vectors have widespread tropism for many tissues. Gene expression by first generation vectors, however, is limited by the induction of neutralising antibody responses against the viral capsid proteins and cytotoxic T-cell responses directed against transduced cells.²⁰³ The complex nature of the adenoviral genome makes deletion of further viral genes problematic and, unlike lentiviral vectors, the development of a packaging cell line has not been possible. Helper-dependent systems have been developed to produce 'gutless' Ad vectors that contain only the viral inverted terminal repeats and the packaging signal, and these vectors can accommodate DNA inserts of up to 28-32 kb. Although these vectors do mediate more sustained transgene expression,²⁰⁴ they do not evade humoral immune response to the viral capsid proteins and even gutless vectors lead to the induction a cellular immune response.²⁰⁵ Since the Ad genome does not integrate into the host chromosome, but remains purely episomal, expression of transgene in the long-term is likely to require repeated vector administration. Although there are over 50 different human adenoviral serotypes the majority of current Ad vectors are derived from types - 2 and -5. These are the most common serotypes to which many adults have been exposed, and over 55% of the adult population are seropositive for Ad-5.²⁰⁶ Clinical studies have demonstrated that the extent of the antibody response to adenoviral vectors depends on pre-existing antibody titres and varies according to the route of vector administration.²⁰⁷

Following delivery into the anterior chamber of the eye, Ad vectors efficiently transduce cells of the corneal endothelium²⁰⁸ and trabecular meshwork.^{209,210} Following delivery into the subretinal space, Ad vectors mediate efficient transduction of RPE cells and occasional photoreceptors.^{211,212} T cell-mediated immune responses, however, can cause local inflammatory changes and limit the duration of gene expression in the eye.²¹³ Although intraocular expression may be prolonged by preventing T cell activation, for example by co-injection with adenovirus expressing CTLA4-Ig,²¹⁴ and newer generation vectors may offer more sustained expression profiles, adenoviral vectors are generally better suited to applications in which only short-term expression is adequate or desirable.

1.4.1.2 Recombinant adeno-associated virus vectors

AAV is a small (20-25 nm) human parvovirus that is able to infect both dividing and non-dividing cells. There are 8 known human viral serotypes, each of which appears to have different tropic properties.²¹⁵⁻²¹⁷ The viral genome is single stranded DNA and consists of two separate open reading frames between two inverted terminal repeats (ITRs). One open reading frame encodes the viral capsid proteins (*cap*) while the other encodes proteins required for replication (*rep*). After infection the single-stranded DNA genome is converted into its transcriptionally active double stranded form by the host's DNA repair machinery and preferentially integrates into the human chromosome site-specifically at 19q13.3.²¹⁸

AAV vector genomes may either undergo chromosomal integration or remain episomal. Since the only *cis*-acting elements required for vector production are the ITRs, recombinant AAV vectors need incorporate no viral genes. Although the recombinant vector retains the ability to integrate into the host chromosome it appears to do so less efficiently than the wild-type virus and without its site-specificity.²¹⁹ The integration of vector genome appears to be responsible for the long-term expression *in vivo* that is typical of AAV vectors in many tissues.²²⁰ In a proportion of transduced cells the recombinant genome persists as high molecular weight concatamers.^{221,222}

Although the incorporation of sections of the *rep* gene into AAV appears to restore site-specific integration in human cells *in vitro*²²³ the utility of this must be weighed against the risk of *rep*-induced immune responses against transduced cells.

Although the production of high-titre AAV vector preparations has been limited in the past by technical difficulties, these have been addressed by the development of new production systems. The original method of AAV production required the co-transfection of a vector plasmid and a packaging plasmid (providing *rep* and *cap* *in trans*) into a producer cell line and infection by a wild-type adenoviral helper virus. This system resulted in only modest titres and risked contamination by live helper virus or by structural helper virus proteins even after labour-intensive purification processes. A number of strategies have since been developed to improve the efficiency of AAV vector production. These include the generation of cell lines that contain integrated copies of some or all of the AAV genes required for packaging²²⁴ and the construction of adenovirus helper plasmids that can be used instead of adenovirus infection.²²⁵ Recombinant helper viruses have been engineered to express the rAAV vector genome²²⁶ or the AAV *rep* and *cap* genes.²²⁷ The replication and expression of AAV *rep* and *cap* gene products can be enhanced through the use of herpes-derived amplicons that support the replication of both the packaging plasmid and the vector plasmid, in combination with a herpes simplex helper virus, resulting in the production of higher titres of transducing particles.^{228,229}

Wild-type AAV is non-pathogenic in humans, and recombinant AAV vectors do not appear to induce significant immune responses. Up to 80% of the population are sero-positive for AAV and antibodies against serotype-2 are the most prevalent.²³⁰ In the majority of cases, however, these antibodies are non-neutralising²⁰⁶ and even in the presence of a strong humoral response to the vector, transduction events can occur after re-administration of rAAV.^{221,231,232}

The small packaging capacity of AAV vectors limits the size of the therapeutic transgene and regulatory sequences that can be accommodated to approximately 4.7 kb. Whilst this is adequate for many applications, modifications to the vector system

that enable the incorporation of larger inserts would offer a major improvement. A trans-splicing approach, in which gene expression is reconstituted from two independent AAV vectors might overcome the vector's packaging limitations²³³ but the success of this system to date has been limited. The efficiency of trans-splicing appears to be serotype dependent,²³⁴ however, and may be improved by the selection of appropriate AAV serotypes; the use of AAV-2 pseudotyped with the capsid of AAV-5 (AAV-2/5), for example, increases the efficiency of trans-splicing in photoreceptor cells.²³⁴

AAV-2 vectors efficiently transduce rod photoreceptors and RPE cells after a single sub-retinal administration,^{235,236} resulting in stable and long-lasting transgene expression.^{237,238} Cells transduced by AAV show a characteristic time delay between exposure to vector and onset of gene expression that has been attributed to the conversion of the incoming single stranded DNA genome to a transcriptionally active double-stranded template.²³⁹ One strategy to alleviate the requirement for host-cell DNA synthesis is through the use of self-complementary AAV vectors.²⁴⁰ When the genome is half wild-type size, AAV can package either two copies, or dimeric inverted repeat DNA molecules that spontaneously re-anneal resulting efficient transduction independently of DNA synthesis. While half-length AAV may accelerate the onset of transgene expression, the size limitation may make it impractical for many disease applications.

AAV pseudotyping provides a useful means to manipulate cell targeting specificity and transduction characteristics in the retina.^{241,242} In the retina AAV-2 mediated gene expression occurs as early as three days after vector delivery and reaches its maximum potential after several weeks.²⁴³ AAV-5 pseudotyped vectors display faster expression kinetics than AAV-2 and mediate greater transduction efficiency.^{242,244} These differences are believed to reflect differences in the relevant target cell receptors and co-receptors; AAV-2 receptors include heparan sulfate proteoglycan, FGF receptor 1 and $\alpha(v)\beta_5$ integrins whereas the AAV-5 receptor complex includes 2,3 linked sialic acid. AAV pseudotyping can significantly alter cell targeting specificity as well

as expression profiles in the retina. For example, AAV-2/6²⁴² and AAV-2/4 (F.Rolling, personal communication) specifically target RPE cells while AAV2/3 fails to transduce retinal cells altogether.²⁴²

Despite their delayed onset of expression and small insert capacity, AAV vectors are attractive candidates for the sustained, non-pathogenic transduction of non-dividing cells for a broad range of applications in the eye and other organs.

1.4.1.3 Retroviral and lentiviral vectors

Retroviruses are RNA viruses that encode reverse transcriptase and are able to transduce cells only during mitosis, integrating into the host genome. Since retroviruses do not transfect non-dividing cells, their useful application in the eye is limited to the targeting of abnormal cell division. The transduction specificity of retroviral vectors for dividing cells has been exploited to target abnormally proliferating cells in proliferative vitreoretinopathy^{245,246} posterior capsular opacification²⁴⁷ and retinoblastoma.²⁴⁸

Lentiviruses are a subfamily of the retrovirus that includes human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV) and equine infectious anaemia virus (EIAV). Like retroviruses, their genomes are integrated into the host genome and stably expressed for a long period but lentiviruses have the additional ability to stably transduce non-dividing and terminally-differentiated cells. This feature has been attributed to several of the viral proteins that are purported to be involved in the nuclear localization of the pre-integration complex.²⁴⁹ Nuclear import is also augmented by a unique triple stranded DNA region created during reverse transcription of the incoming viral RNA in the target cell.²⁵⁰ Lentiviral vectors can deliver and integrate more than 8 kb of transgenic DNA into target cell genomes. Since they are deleted for potentially pro-inflammatory viral components, multiply-attenuated vectors do not induce a significant host immune

response against transduced cells but retain the ability to mediate efficient gene delivery *in vivo*.²⁵¹

One concern regarding HIV-derived vector systems is the possibility of inadvertently generated replication-competent retrovirus during vector production. The risk of recombination may be reduced by minimizing HIV sequences on the vector and on the helper sequences, and by production using multiple plasmid expression systems.²⁵² The possibility that the lentiviral long terminal repeat (LTR), critical to genome integration, might drive transcription of an unwanted viral gene has been addressed by the development of self-inactivating vectors.^{253,254} These vectors have a deletion in the 3' LTR that is transferred to the 5' LTR after reverse transcription and integration in infected cells, resulting in the transcriptional inactivation of the LTR, thus abolishing its promoter activity. This system reduces the likelihood that replication competent retroviruses will originate in the vector producer and target cells, and additionally improves the potential performance of the vector by removing LTR sequences previously associated with transcriptional interference and suppression *in vivo*.

The development of non-primate vectors, such as those based on FIV²⁵⁵ and EIAV^{256,257} may offer safer alternatives to HIV-based vectors since these viruses are not known to cause human disease. The use of non-primate lentiviral vectors may also ease potential psychological barriers to the introduction of lentiviral vectors in clinical settings.

To expand their tropism beyond CD4-positive cells, lentiviral vector particles have been pseudotyped using a range of envelope proteins. Vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped lentiviral vectors²⁵⁸ have proven useful for stable gene transfer into human haematopoietic stem cells, terminally differentiated macrophages and neuronal cells.²⁵³ Vector pseudotyping with rabies virus glycoprotein enables targeting of peripheral neurones and retrograde axonal transport.²⁵⁹

In ocular tissues, transduction profiles of a number of lentiviral vectors have been investigated. RPE cells are efficiently transduced following subretinal delivery of HIV-

1,^{260,261} FIV²⁶² and SIV²⁶³ vectors, though transduction of photoreceptors is relatively inefficient. In contrast to AAV vectors, lentiviral vectors mediate efficient transgene expression as soon as 4 days after subretinal administration and maximum transgene expression is reached within 3 weeks.²⁶³ Lentiviral vectors also mediate efficient and sustained transgene expression in corneal endothelial cells *in-vitro*, *in-situ*²⁶⁴ and following anterior chamber injection *in-vivo*.²⁶¹

1.4.1.4 Non-viral vectors

The administration of non-viral vectors can result in transduction of cells in a number of ocular tissues but gene expression is generally inefficient and short-lived compared to virus-based alternatives.²⁶⁵ Ballistic gene transfer to the cornea has been attempted but this approach is complicated by significant tissue injury.²⁶⁶ Targeted gene transfer to corneal endothelium can be facilitated by the application of an electric pulse.²⁶⁷ Cationic liposomes are able to mediate sustained intravitreal delivery of oligonucleotides²⁶⁸ and may be used as vehicles for ocular gene transfer²⁶⁹ but expression is short-lived compared to virus-based vectors.

1.4.2 Factors influencing ocular gene transfer

The pattern of cell transduction and efficiency of gene expression following intraocular delivery of a given vector is determined by a number of variables including the route of administration, the vector serotype and the regulatory sequences incorporated into the expression cassette of the transgene. Since ocular tissues are highly compartmentalized, the pattern of tissue transfection by a given vector is dependent on the site of its intraocular administration. Delivery of AAV vectors into the subretinal space, for example, results in the transduction of photoreceptors and retinal pigment epithelial cells whereas injection of the same vector into the vitreous targets only ganglion cells, at least in the adult eye.²⁷⁰ Delivery of Ad vectors into the subretinal space mediates gene transfer to the RPE and very occasional photoreceptor cells²¹¹ but

when delivered to the vitreous or anterior chamber transduces corneal endothelium.²⁰⁹ Intravitreal delivery of Ad vectors also results in transduction of retinal Muller cells and this is more efficient when a vitrectomy is first performed.²⁷¹ The capsid proteins of different vector serotypes confer differences in cell tropisms as discussed above (section 1.4.1.2). AAV serotypes -4 and -5 appear to be more efficient than serotypes 1-3 for the transduction of retinal cells and their resulting expression is more rapid in onset.²⁴⁴ Pseudotyping of vectors with different capsid proteins provides a useful means to manipulate viral vector cell targeting specificity as well as retinal transduction characteristics of vectors containing the same genome.²⁴¹ Selection of the transgene promoter is critical in determining the efficiency and specificity of expression by a transduced cell. While a CMV promoter, for example, ubiquitously drives efficient expression in all transduced cells, tissue-specific promoters can restrict expression to a given cell type.

For these reasons the optimal choice of vector construct, promoter sequences and route of delivery for any given application is determined by the nature of the target cell and the desired timecourse of transgene expression.

1.4.3 Applications of ocular gene transfer

Ocular gene transfer strategies have been described for inherited disorders, expression of protective factors and apoptosis modulators in degenerative disease, introduction of so-called "suicide genes" in proliferative diseases and expression of immuno-modulatory factors in immune disorders.^{272,273}

Significant success has been achieved using gene replacement therapy in experimental models of recessively inherited retinal degeneration. In studies that demonstrated for the first time that a complex ultrastructural cell defect can be corrected both morphologically and functionally by *in vivo* gene transfer, AAV-mediated replacement of the structural protein peripherin/rds in retinal degeneration slow (rds) mice restored structure and function of photoreceptor cells.²⁷⁴ Efficient gene replacement however,

may not be sufficient to arrest retinal degeneration despite the improved morphology and function; although the potential for ultrastructural improvement was dependent upon the timing of intervention in rds mice, there was no significant effect on photoreceptor cell loss in this model, irrespective of the date of vector administration.²⁷⁵ Accurate control of gene expression as well as early intervention may be critical to this approach. A combined strategy involving gene replacement with expression of a survival factor may also offer an improvement in the timecourse of retinal degeneration.

Gene replacement strategies in experimental models of inherited enzyme deficiencies have also resulted in the amelioration of retinal degenerations. Delivery of a normal beta subunit of the cGMP phosphodiesterase gene (beta PDE) in retinal degeneration (rd) mice by Ad-mediated gene transfer delays photoreceptor cell death.²⁷⁶ In the Royal College of Surgeons (RCS) rat, Ad-mediated gene delivery of functional *Mertk* to the RPE restores its ability to phagocytose shed photoreceptor outer segments and leads to preservation of photoreceptor cells.²⁷⁷ In a canine model of Leber's Congenital Amaurosis (LCA), the RPE65^{-/-} dog, subretinal injection of AAV vectors encoding RPE65 results in the restoration of visual function both electrophysiologically and behaviourally.^{278,279}

Proof of principle has been also been presented for treatment of dominantly inherited retinal degenerations by intraocular delivery of genes encoding ribozymes, catalytic RNA molecules that cleave complementary mRNA sequences. For example, the rate of photoreceptor loss in a rat model of dominantly inherited retinal disease is slowed by expression of a ribozyme that catalyzes the destruction of the mutant mRNAs.²⁸⁰ Similar approaches have been described for the rescue of photoreceptor cells in P23H transgenic rats²⁸¹ and of a porcine model of autosomal dominant retinitis pigmentosa.²⁸²

An alternative gene therapy strategy that may be broadly applicable to a number of retinal degenerations is the expression of neurotrophic factors in an attempt to delay neuronal degeneration.²⁸³ Glial cell line-derived neurotrophic factor (GDNF) induces

histologic and functional protection of rod photoreceptors in the rd/rd mouse²⁸⁴ and delays photoreceptor degeneration in a transgenic rat model of retinitis pigmentosa.²⁸⁵ A number of studies have demonstrated a beneficial effect of vector-mediated ciliary neurotrophic factor (CNTF) gene expression on photoreceptor cell loss. Although CNTF expression can delay photoreceptor degeneration,²⁸⁶ however, the morphological improvement is not associated with improved function.²⁸⁷ Indeed AAV –mediated expression of CNTF results paradoxically in significant loss of retinal function despite preservation of morphology in the retinal degeneration slow (rds) mice and also reduces visual function in wild-type mice.²⁸⁸ The mechanisms that underly these observations are not well understood but may involve de-differentiation of photoreceptor cells or remodelling of the inner retina.

Ocular disorders involving cell proliferation have been targeted by a ‘suicide gene’ approach in which delivery of a gene encoding an enzyme leads to death of transduced cells following its activation. This strategy has been applied in the eye to destroy lens epithelial cells in posterior capsule opacification,^{289,290} RPE cells in proliferative vitreoretinopathy^{245,291} and tumour cells in retinoblastoma.²⁴⁸ Transduction of herpes simplex virus-thymidine kinase is associated with a powerful bystander effect both *in vitro* and *in vivo* with significant effects even when transduction efficiency is low.²⁴⁶ An alternative approach to target proliferating cells is the delivery of antisense oligonucleotides that suppress gene expression by specific binding to complementary mRNA molecules. These molecules have a short half-life in the vitreous and require repeated administration but their sustained expression can be achieved by a gene transfer approach. For example, delivery of genes encoding antisense to cyclin G1 and MAT1 inhibits rabbit keratocyte and human fetal lens epithelial cell proliferation²⁴⁷ Alternative strategies to inhibit cell proliferation include retrovirus-mediated transfer of cell cycle control genes,²⁴⁷ and the introduction of tumour suppressor genes.²⁹²

1.4.5 Strategies for angiostatic gene transfer

A gene transfer approach to angiogenesis offers the potential means to interrupt its molecular pathways by the targeting of relevant factors at a number of levels, from upstream processes such as transcription, through to cytokine and extracellular matrix interactions, to downstream intracellular effector mechanisms. The transcription of pro-angiogenic cytokines may be inhibited by vector-mediated expression of antisense DNA as has been described for the targeting of hypoxia-inducible factor-1 (HIF-1),²⁹³ VEGF,²⁹⁴ angiostatin²⁹⁵ and of integrins.²⁹⁶ Another approach is the expression of ribozymes, catalytic RNA molecules that cleave complementary mRNA sequences, as has been described in the targeting of VEGF²⁹⁷ and of its receptor Flt-1.²⁹⁸ The action of pro-angiogenic cytokines might be inhibited by the vector-mediated expression of appropriate protein inhibitors. The interaction of VEGF with its endothelial cell receptors, for example, may be inhibited by the expression of the VEGF inhibitor sFlt-1. Alternatively angiogenesis may be opposed by the overexpression of endogenous angiostatic factors such as endostatin²⁹⁹ and angiostatin.³⁰⁰

An alternative gene transfer strategy to molecular targeting is to mediate the destruction of critical cellular mediators of angiogenesis such as proliferating vascular endothelial cells. One novel strategy, for example, is based on the targeting and preferential killing of proliferating endothelial cells by the delivery of the herpes simplex virus thymidine kinase gene (HSV-TK) under the control of a von Willebrand factor (vWf) promoter leading to preferential suppression of endothelial cell growth in the presence of the prodrug ganciclovir.³⁰¹

The optimal vector for a given angiostatic gene transfer strategy will depend on the nature of the cell to be targeted and the desired timecourse of expression. Antisense and ribozyme strategies, for example, are likely to require highly efficient transduction of a range of cell types responsible for the expression of angiogenic factors. Expression of soluble angiostatic factors, on the other hand, need not require transduction of the endogenous cell source or of the target cell, but may be mediated by transduction of

any cell types that results in therapeutic concentrations. Since both rAAV and lentiviral vectors are able to mediate sustained expression by non-dividing cells these are attractive candidates for this purpose.^{126,302} Although adenoviral vectors have been used successfully to express angiostatic factors in cancer gene therapy³⁰³⁻³⁰⁵ the relatively short-lived expression mediated by these vectors may not offer the sustained effect that is expected to be required in the successful management of ocular neovascular disorders. Whilst retroviral vectors may be used to target proliferating vascular endothelial cells during periods of active angiogenesis,³⁰⁶ this approach is unlikely to offer protection against neovascularisation in the long term.

1.5 Aims of the thesis

The purpose of the experiments described in this thesis is to investigate whether local angiostatic gene transfer can effectively and safely control neovascularisation in the retina and choroid. The thesis describes the development and optimisation of experimental models of retinal and choroidal neovascularisation in which to test this hypothesis. The efficiency of gene delivery is characterised in these models by the investigation of vector-mediated reporter gene expression. The potential therapeutic effect of angiostatic gene transfer is determined by quantification of the extent of neovascularisation following treatment and the potential for adverse effects investigated in normal animals. The possibility of adverse effects may be minimised by appropriate regulation of gene expression and for this purpose the effect of a tissue-responsive promoter is determined in models of both choroidal and retinal neovascularisation. Pre-clinical studies to investigate the efficiency and potential toxicity of reporter gene transfer in large animals are described. Having demonstrated proof of principle that angiostatic gene transfer can be an effective approach in experimental models of retinal and choroidal neovascularisation a proposal for a clinical trial of angiostatic gene transfer is presented.

2

Materials and methods

2.1 Reagents

All reagents were from Merck Ltd., Leics, UK unless otherwise stated. Recipes for solutions are given in section 2.14.

2.2 Amplification of plasmid DNA in bacteria

2.2.1 Transformation of competent cells

For transformation, DH5 α [™] competent cells (Invitrogen Ltd., Paisley, UK), or Epicurian Coli[®] SURE[®] 2 competent cells (Stratagene Europe, Amsterdam, Netherlands), were thawed on ice. A 50 μ l aliquot was incubated with the plasmid DNA (20 minutes on ice) before heat shock at 42 °C in a pre-warmed water bath for 90 seconds followed by 90 seconds on ice. LB (Luria-Bertani) medium 500 μ l at room temperature was added to the mix, and incubated with gentle shaking at 37 °C for 30 minutes. The suspension was centrifuged at 4000 rpm for 1 minute. The pellet was re-suspended in 50 μ l LB medium and spread on a LB-agar plate, containing 50 μ g/ml ampicillin. Plates were incubated overnight at 37 °C to allow for the growth of resistant colonies.

2.2.2 Amplification and recovery of recombinant plasmid DNA

Bacterial colonies from agar plates were inoculated into 5 ml of LB media containing 50 μ g/ml ampicillin and incubated at 37 °C with agitation for 12-16 hours. For small-scale preparations, nucleic acid was prepared using a QIAprep[®] Spin Miniprep Kit (QIAGEN Ltd., W. Sussex, UK). For bulk cultures, 200 μ l of the 5 ml culture was used

to inoculate a further 250 ml of LB, which was incubated as above. Plasmid DNA was recovered with a QIAGEN® Plasmid Mega Kit (QIAGEN Ltd.).

2.2.3 Quantification of nucleic acid

Nucleic acids produced with the QIAGEN Mega Kits were quantified using a Unicam UV 500 spectrometer. An absorption of 1 at OD_{260nm} was taken to equal a concentration of 50 µg/ml double stranded DNA. Nucleic acids produced with QIAGEN Mini Kits were quantified on a 1% agarose gel (section 2.3.2).

2.3 DNA analysis

2.3.1 Restriction enzyme digestion of plasmid DNA

Digestion of DNA was carried out in accordance with enzyme manufacturer's instructions, in a 1 x buffer with an excess of enzyme (5-10 U/µg DNA).

2.3.2 Electrophoresis of DNA

DNA products were separated on a 0.8 – 1.2 % (w/v) agarose gel with 1% (w/v) ethidium bromide using a 1 x TAE buffer. A 1 kb DNA ladder (Invitrogen Ltd.) was run to provide size markers. Samples were loaded using gel-loading buffer. Gels were run using the voltage and duration for the separation of the required DNA bands. Gels were photographed on a UVP ultraviolet (UV) transilluminator. The approximate concentration of DNA was assessed through comparison with the 1636 bp band from the DNA ladder, which contains 10% of the mass applied to the gel.

2.4 Cloning in plasmid vectors

2.4.1 Creating appropriate DNA fragments

DNA fragments that were required for cloning protocols were generated with restriction enzyme digests (section 2.3.1). For cloning into plasmids, where possible,

non-complementary ends were used to enhance the efficiency and determine orientation of the insertion. Otherwise, to prevent intra-molecular ligation, the vector molecule was pre-treated with calf intestinal alkaline phosphatase (New England Biolabs (UK) Ltd.) to remove 5'-phosphate groups. One microlitre was added after the restriction digest and the mixture incubated at 37 °C for 1 hour. Where a blunt ligation was necessary the 5' overhang was filled using 1 µl of Klenow fragment DNA-polymerase I (New England Biolabs (UK) Ltd.) in the presence of excess (0.1 mM) deoxynucleotide triphosphates (dNTPs – Promega UK, Southampton, UK), both added after the restriction enzyme digest and incubated at room temperature for 15 minutes. These reactions were terminated by the addition of gel loading dye. Samples were run out on an agarose gel (section 2.3.2) and the required bands excised with a scalpel under ultraviolet light.

2.4.2 Isolation of DNA fragments from agarose gels

DNA fragments excised from agarose gels were extracted from the agarose using QIAquick™ Gel Extraction Kits (QIAGEN Ltd.). DNA was eluted from the column using 30 µl of TE buffer (supplied by manufacturer). The concentration of the eluted sample was assessed by comparing 5 µl of the sample against the 1636 bp band of the 1 kb DNA ladder (Invitrogen Ltd.) on an agarose gel as described in section 2.3.2.

2.4.3 DNA ligation

The ligation of gel-purified DNA fragments was performed at 16 °C for 4-16 hours, using T4 DNA ligase (New England Biolabs (UK) Ltd.) with buffer at concentrations recommended by the manufacturer and at a vector to insert molar ratio of 3. Where oligonucleotides were ligated into plasmids they were used in excess (100 µM). Where PCR fragments were ligated into pGEM®-T Easy plasmids (Promega UK) ligations were performed according to manufacturer's instructions.

2.4.4 Confirmation of successful plasmid ligation

A 2 µl sample of the ligation mixture was transformed and amplified in bacteria (section 2.2) and successful ligation determined by restriction digestion analysis of purified plasmid DNA (section 2.3).

2.5 Polymerase chain reaction (PCR)

PCR reactions were performed in a volume of 50 µl using 0.2 mM dNTPs (Promega UK), 25 µM of each primer, and 1 unit of enzyme. The PCR was carried out using the proof reading enzyme *Pfu* polymerase (Stratagene, Amsterdam, Holland) according to the manufacturer's instructions. The buffer used was that supplied by the manufacturer. The reaction was performed in triplicate at three different MgCl₂ concentrations (0.5, 1.5 and 2.5 mM) with an additional control reaction to which no template DNA was added. The amount of template DNA was approximately calculated to provide >10⁴ copies of the target sequence, but the final DNA concentration of the reaction was kept to less than 10 ng/µl. Reactions were temperature cycled using a Techne Touchgene Thermal Cycler. All the reaction cycles were based around the following:

Initial denaturation	3 minutes at 94 C	
Denaturation	1 minute at 94 C	} 30 cycles (35 cycles for PCR on genomic DNA)
Annealing	1 minute at X C	
Extension	X minutes at 72 C	
Final extension	10 minutes at 72 C	

The temperature of annealing for each set of primers was calculated by using the program PrimerSelect™ (DNASar Inc.). During the extension step 1 minute was allowed for every kb of amplicon (minimum extension time = 1 minute). Where the primers incorporated restriction enzyme overhangs 5 cycles were inserted prior to the main batch of 30 in which the annealing temperature corresponded to that of the primer without the overhang. The annealing temperature of the entire primer was used in the

main batch of 30 cycles. Ten microlitres of each PCR reaction was analysed on an agarose gel (section 2.3.2).

2.6 Tissue culture

2.6.1 Cell lines and viruses

The baby hamster kidney (BHK) cell line was obtained from European Collection of Animal Cell Cultures (ECACC, Porton Down, UK). The disabled single-cycle herpes simplex (DISC-HSV) helper virus (DISC-HSV), PS1, used in the production of AAV was prepared by Ajay Mistry.

2.6.2 Culture of cell lines

BHK cells were grown in BHK-21 media (Invitrogen Ltd.) supplemented with 5 ml of antibiotic-antimycotic (Invitrogen Ltd.), 10% heat inactivated foetal calf serum (FCS - Invitrogen Ltd.), 5 % tryptose phosphate broth (Invitrogen Ltd.), 5 ml of 200 mM L-glutamine (Invitrogen Ltd.) and 4 ml of 50 mg/ml Geneticin (Invitrogen Ltd.). Cells were grown in a Sanyo CO₂ incubator at 37 °C with 5% CO₂.

2.6.3 Splitting and counting cells

Prior to passaging plates of BHK cells were washed twice with PBS. The cells were then incubated with a thin covering (approximately 1.5 ml for 15 cm dishes) of trypsin-EDTA (Invitrogen Ltd.) for 10 minutes at 37 °C. The trypsinisation was stopped with the addition of 10 volumes of growth medium and the cells were split one in six. These cells were split every 2-3 days and were not allowed to overgrow. Approximate numbers of cells were counted using a haemocytometer.

2.6.4 Long-term storage of cells

Cells were frozen at approximately 1×10^6 cells/ml in growth media with 20% FCS (Invitrogen Ltd.) and 10% DMSO (Invitrogen Ltd.) by first cooling slowly to -70 °C overnight in a polystyrene box containing pre-cooled isopropanol. The next day cells

were transferred to liquid nitrogen for long-term storage. To thaw, cells were warmed quickly in a 37 °C water bath, diluted in growth media, pelleted by centrifugation and resuspended in the appropriate medium.

2.7 Manufacture of replication defective AAV

Production of AAV

For AAV production, BHK cells were plated at 4×10^6 cells/150 mm dish 1 day prior to transfection so that they were approximately 70-80% confluent on the day of transfection. Twenty dishes were routinely used for large-scale preparation of virus. Cells were transfected with Lipofectin/Integrin targeting peptide/DNA (LID) transfection complexes. These were made by gentle mixing of three components: cyclised integrin targeting peptide 6 (Zinsser Analytic, Maidenhead, UK) at 0.1 mg/ml in Opti-MEM® (Invitrogen, Ltd.), Lipofectin (Invitrogen, Ltd) and plasmid DNA (prepared as in section 2.2), in a weight ratio of 0.75:4:1. The order in which these components are mixed is important for the efficiency of transfection. A total of 60 µg of DNA was transfected into a single 150 mm dish; this total consists of a 1:3 weight ratio of vector plasmid : helper plasmids. The appropriate amounts of each plasmid were mixed and then made up to 6 ml with Opti-MEM®. In a separate tube 45 µl of Lipofectin (1 mg/ml) was added to 6 ml of Opti-MEM®, and then 2.4 µl of integrin targeting peptide (0.1 mg/ml) was added and mixed gently. The DNA was added to Lipofectin and peptide and then mixed by inverting the tube. Complexes were allowed to form (1 hour at room temperature). Cells were washed twice with Opti-MEM® medium and then each dish was covered with 12 ml of Lipofectin/Integrin targeting peptide/DNA complexes mixture. The plates were incubated at 37 degrees C under normal 5 % CO₂ conditions for 5 hours. The transfection media was removed and normal growth media was added containing DISC-HSV (PS1) at 10-20 infectious units per cell. Cells were incubated for a further 32-35 hours. During this period the cells were monitored as the cytopathic process occurred. The cells were harvested by

scraping just as they started to lift off from the plate, and they were pelleted by centrifugation (1200 rpm for 10 minutes).

2.7.2 Purification of AAV

Cells from 10 plates were re-suspended in 15 ml of TMN buffer. Cells were lysed through three cycles of freeze thawing between dry ice-ethanol and a 37 °C water bath, with vortexing between each cycle. Genomic DNA was removed by digestion with Benzonase, (50 Units per 10 plates), incubated at 37 °C for 30 minutes. The lysate was clarified by centrifugation (3700 g for 20 minutes), and the virus-containing supernatant retained. Sodium deoxycholate was added to the viral supernatant to give a final volume of 0.5% and the solution incubated for 30 minutes at 37 degrees C. This solution was then was put through a 5 µm filter and then a 0.8 µm filter (Millipore Corporation, MA, USA) to remove any large particles, such as the DISC-HSV. The AAV was further purified through a 2.5 ml pre-packed Heparin-Agarose column (Sigma-Aldrich Company Ltd.). The existing column buffer was allowed to drip through the column before equilibration with 4 x 5 ml of 1 x PBS-MK. The 10 ml of filtered virus solution (section 2.8.2) was then applied to the column under gravity and allowed to pass through. The column was washed with 10 ml of PBS-MK before elution of the virus through the addition of 6 ml of PBS-MK/1 M NaCl solution. After applying this elution buffer the first 2 ml of the eluate was discarded as dead volume.

Concentration of AAV

The AAV within the eluate was concentrated and desalted in a Centricon YM-10 filter device (Amicon Bioseparations, Millipore Corporation), according to the manufacturer's instructions. The eluate was split between two filter devices that were then spun at 4000 g for 50 minutes. The high-salt buffer was exchanged by repeatedly diluting the concentrated virus with storage buffer. The elution was discarded and a further 2 ml of storage buffer is added to each before spinning again at 4000 g for 50 minutes. The concentrated virus (approximately 150 µl) was then pooled and stored at -70 °C.

2.8 Titration of AAV preparations

2.8.1 Extraction of viral DNA from AAV particles

Two aliquots of 1 or 5 μl were taken from the AAV produced in section 2.8. These samples were digested with 100 μg of proteinase K (Promega UK) in a volume of 400 μl with 1 x proteinase K buffer, to liberate the AAV genomic DNA from the capsid proteins. This digest was incubated at 37 °C for 1 hour. The contaminating proteins were removed through phenol extraction. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was thoroughly mixed with the solution and centrifuged for 13000 rpm for 5 minutes to separate organic and aqueous layers. DNA was precipitated from the aqueous layer through the addition of 40 μg of glycogen (Sigma-Aldrich Company Ltd.), 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol, and incubation at -70 °C for 30 minutes. The DNA was pelleted by centrifugation for 13000 rpm at 4 °C for 30 minutes. The resulting pellet was washed with 70% ethanol and then re-spun as above for 20 minutes. The pellet was air-dried and resuspended in 20 μl of dH_2O .

2.8.2 Preparation of dot blot

A standard curve was prepared through the serial dilution of a plasmid containing the section of DNA that was to be used as a probe. Routinely eight samples were prepared ranging from 10^{12} molecules to 10^5 molecules in 10 fold serial dilutions. These were prepared in dH_2O . These samples, and those from section 2.9.1 containing the recombinant viral genomes, were denatured by the addition of 10 μl of alkaline stock solution (10x) with 70 μl of dH_2O , and heating at 100 °C for 5 minutes. Samples were then kept on ice to prevent re-annealing. Dot blots were prepared on pre-wetted 0.45 μm Hybond™ - N+ membrane (Amersham Pharmacia Biotech. UK, Bucks, UK) in a dot blot manifold (Bio-Rad Laboratories Ltd., Herts, UK). The wells of the manifold were pre-washed with 0.4 ml of dH_2O and the vacuum applied until they were empty. The denatured DNA was added without vacuum and then the vacuum applied. The wells were rinsed with 0.4 ml of alkaline stock solution (1x). The membrane was

removed from the manifold and washed in sodium phosphate buffer (pH 7.2), dried between blotting paper and stored at room temperature.

2.8.3 Preparation of probe

The section of DNA to be used as the probe was excised from the plasmid with a restriction enzyme digest (section 2.3.1), separated on a gel (section 2.3.2) and then extracted from a gel fragment (section 2.4.3). A Prime-It® II Random Primer Labelling Kit (Stratagene Europe) was used to label the probe radioactively with $\alpha^{32}\text{P}$ dCTP (Amersham Pharmacia Biotech, UK) according to manufacturer's instructions. Unincorporated dNTPs were removed using a MicroSpin™ G50 Column (Amersham Pharmacia Biotech, UK). Immediately before use the probe was denatured at 96 °C for 2 minutes.

2.8.4 Hybridisation of membrane

The dried membrane was wetted in dH_2O , rolled with gauze and then placed in a hybridisation tube with room temperature 0.3 M sodium phosphate buffer. The tube and its contents were heated to 65 °C in a hybridisation rotisserie oven. Once at 65 °C the buffer was exchanged for Church buffer (pre-warmed to 65 °C) and the membrane pre-hybridised for 30 minutes. The radioactively labelled probe (section 2.8.3) was then added to the Church buffer and the membrane hybridised for 5 hours. The membrane was washed with a ten-fold dilution of sodium phosphate buffer (pH 7.2), twice at room temperature (2 minutes) and twice at 65 °C (10 minutes) with pre-warmed buffer. Excess liquid was removed and the membrane sealed in plastic. The membrane was then placed in a cassette with Kodak Scientific Imaging BioMax™ MR-1 film (1-4 hours at room temperature), which was later processed in a Fuji RG II X-Ray Film Processor. The titre of the AAV preparation was determined by comparison with the standard curve of reference plasmid.

2.9 Manufacture of HIV-1 based lentiviral vectors

The production of VSV-G pseudotyped vectors were produced by transient transfection of three plasmids into 293T cells.²⁵⁴ The transfer vector was generated by modification of a basic self-inactivating vector pHR'SIN-CE (which itself incorporates a CMV promoter and enhanced green fluorescent protein (eGFP) reporter gene²⁵⁴) with the woodchuck post-transcriptional regulatory element (WPRE), and the central polypurine tract cis-acting sequence (cPPT) and central termination sequence (CTS) of HIV-1. The WPRE was first rescued from pBlueScript-WPRE-B11 (gift from Thomas J. Hope, Salk Institute, La Jolla, CA) and inserted downstream of the eGFP stop codon in pBlueScript containing the eGFP open reading frame. Subsequently, pHR'SIN-CEW was constructed by substitution of the BamH1–Xho1 eGFP cassette of pHR'SIN-CE with eGFP-WPRE. The 178 bp fragment, encompassing the central polypurine tract, was amplified by PCR from pCMVR8.91 (gift from Pierre Charneau, Insitute Pasteur, Paris) and inserted upstream of the expression cassette to create the final vector pHR'SIN-cPPT-CEW.²⁵⁰ For production of recombinant virus, a total of 10⁷ 293T cells were seeded in one 150 cm² flask over-night prior to transfection. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS), penicillin (100 IU/ml), and streptomycin (100 µg/ml) in a 5% CO₂ incubator, and the medium was changed 2 hours prior to transfection. A total of 100 µg of plasmid DNA was used for the transfection of one flask: 17.5 µg of the envelope plasmid, 32.5 µg of packaging plasmid, and 50 µg of transfer vector plasmid were pre-complexed with 0.25 mM PEI (22 kDa) in 10 ml Optimem at room temperature for 15 min. The DNA plus PEI complexes were then added to the cells. After 4 hours incubation at 37 °C in a 5% CO₂ incubator, the medium was replaced by fresh DMEM supplemented with 10% FCS. Virus particles were concentrated 20 to 100 fold by ultra-centrifugation at 50,000 g for 90 min at 4°C. The pellet was resuspended in serum-free X-VIVO10 medium (BioWitthaker Ltd.) and kept at –80 degrees C until use.

Lentivirus vector titres were determined by transduction of HeLa cells, and for the studies described here, were approximately 2×10^8 transducing units per ml. Lentiviral production and titration were performed by Ajay Mistry.

2.10 Western blot for demonstration of *sFlt-1* expression by AAV-CMV-*sFlt-1* in vitro

293 cells were cultured in 6-well culture plates with DMEM (Gibco BRL) supplemented with 10% fetal bovine serum. The cells were allowed to grow until they reached approximately 80% confluence at which point they were transfected by either AAV-CMV-*sFlt1* (2 μ l/well, 5×10^{10} particles) or by AAV-CMV-GFP (2 μ l/well, 5×10^{10} particles) as control. After 48 h, the conditioned medium was collected and the proteins greater than 10 kDa were concentrated using a Centricon YM-10 (Millipore, Bedford, MA). Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad). Protein samples (50 μ g) were solubilised in SDS-loading buffer and subjected to SDS-polyacrylamide (10%) gel electrophoresis. Protein was transferred onto a nylon membrane (Millipore) and the blot was sequentially incubated in primary antibody (1:500 goat anti-*sFlt*, R&D Systems, Abingdon, UK) and secondary antibody horseradish peroxidase-conjugated rabbit anti-goat (1/1000 Dako). The ECL kit (Amersham) was used to generate a chemiluminescent signal.

2.11 Enzyme-linked immunosorbant assay for quantification of AAV-mediated expression of *sFlt-1* in vivo

Enucleated mouse eyes were washed 3 times in phosphate buffered saline. Eyes were pooled and manually ground in an Eppendorf in 150 μ l of assay buffer with proteinase inhibitors. The resulting suspension was centrifuged at 4000 rpm for 5 mins and the

supernatant assayed for total sFlt-1 using a total sFlt-1 Test Kit (RELIATech, Braunschweig, Germany) as described in Chapter 2; a mouse monoclonal anti-sFlt-1 antibody supplied by the manufacture was used to capture sFlt-1 from the samples and a biotinylated, polyclonal rabbit antiserum was used to detect bound sFlt-1. The OD of samples was compared to a standard curve of recombinant sFlt-1.

2.10 Mouse fundus imaging

2.10.1 Animals

All animals were managed in accordance with the Animal Scientific Procedures Act 1986 and procedures were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.10.2 Mouse fundus photography

Animals were anaesthetised by intraperitoneal injection of hypnorm and hypnovel mixed 1:1:6 with water. Pupillary dilatation was achieved using a drop of topical tropicamide 1% or atropine 1%. A Kowa Genesis small animal fundus camera was used to examine and to photograph the fundi. The instrument was mounted on a clamp stand for stability and used in conjunction with a 90 dioptre (Volk) condensing lens mounted on the same clamp stand between the camera and the eye. A foot pedal was used to operate the shutter thus permitting the use of both hands for handling the animal. One hand was used to support the animal and two fingers of the other to retract the eyelids. The animal was held on its side on the microscope platform approximately 7.5 cm beneath the lens. The animal was positioned to optimise focus, to minimise unwanted light reflections and to facilitate imaging of different parts of the fundus. Kodak Elite 200 ASA slide film was used.

2.10.3 Mouse fundus fluorescein angiography

For fundus fluorescein angiography the standard camera back was replaced with the one specifically manufactured to contain a barrier filter for fluorescein angiography and the powerpack was set for angiography to enable the appropriate integrated excitation filter. The eyepiece was fitted with the manufacturer-supplied barrier filter. Sodium fluorescein 2 % was administered by intraperitoneal injection at a dose of 0.2 ml per 30 g body weight. Photographs were taken at appropriate intervals; the retinal vessels typically begin to fill after 45 seconds and capillary washout occurs approximately 7-8 minutes after dye administration. Provia 400 ASA colour reversal film or Ilford 1600 ASA black and white film was used.

2.11 Intraocular delivery of vector suspensions

Adult mice were anaesthetised by intra-peritoneal injection of 0.2 ml of Hypnorm (Janssen Pharmaceutical Ltd, Oxford, UK) and Hypnovel (Roche, Welwyn Garden City, UK) mixed 1:1:6 with distilled water. Ten-day old pups were anaesthetised with 0.2 ml of a 1 in 10 dilution of the same Hypnorm/Hypnovel mixture.

The palpebral fissure was opened if necessary with a No.11 scalpel blade and the pupil dilated with topical 1 % Tropicamide (1 % Mydracyl, Alcon Labs, Watford, UK). The procedure was performed under an operating microscope. The fundus was visualised by means of a contact lens system consisting of a drop of 1 % hypromellose solution on the cornea, covered with a glass coverslip. A 10 mm 34 - guage needle, mounted on a 5 µl Hamilton syringe (Hamilton, Bonaduz, Switzerland), was used to inject vector suspensions. For intravitreal injections the syringe tip was advanced under direct vision through the sclera, 1 mm posterior to the corneoscleral limbus, into the vitreous and approximately 1 µl of viral suspension was injected into the vitreous cavity. For subretinal injections the needle tip was guided tangentially through the sclera at the equator of the globe to create a self-sealing tunnel into the subretinal space. A volume

of 2 μ l of viral suspension was injected to produce a bullous retinal detachment involving approximately 30% of the fundus.

2.12 Preparation of retinal and choroidal flatmounts

2.12.1 Fluorescein-dextran perfused whole retina flatmounts

Animals were deeply anaesthetised by intraperitoneal injection of pentobarbitone and cardiac perfusion was performed using 1 ml PBS containing 50 mg/ml fluorescein-labelled dextran (2×10^6 average molecular weight, Sigma, St. Louis, MO), clarified by centrifugation for 5 min at 10,000 rpm.³⁰⁷⁻³⁰⁹ The eyes were enucleated and fixed in 4 % paraformaldehyde for 1 hour. The cornea and lens were removed and the retina dissected from the eyecup. The retina was cut radially into four quadrants and flat-mounted in Aquamount under a coverslip for examination by fluorescence microscopy at 200/400 X magnification. Digital photographs were acquired by means of a video camera and frame grabber.

2.12.2 Fluorescein-dextran perfused whole sclera-choroid-RPE flatmounts

Cardiac perfusion was performed using fluorescein-labelled dextran as described above (section 2.12.1). The cornea and lens were removed and the retina dissected from the eyecup. The eyecup comprising the sclera-choroid-RPE was cut radially into four quadrants and flat-mounted in Aquamount under a coverslip for examination by fluorescence microscopy at 200/400 X magnification.

2.12.3 Whole retina flatmounts for detection of GFP expression

Animals were sacrificed by cervical enucleation and their eyes fixed in 4 % paraformaldehyde for 1 hour. The retina was dissected from the eyecup as described above and flat-mounted for examination by fluorescence microscopy at 200/400 X magnification.

2.13 Histological methods

2.13.1 Paraffin wax sectioning

Animals were sacrificed by cervical dislocation and their eyes were immersion-fixed in 4% (w/v) paraformaldehyde (4 hours at room temperature). Eyes were dehydrated overnight in a Leica Histokinette processing machine and embedded in paraffin wax. Sections (6 μ m) were cut using a microtome and stored at room temperature.

2.13.2 Cryo-sectioning

Animals were sacrificed by cervical dislocation and their eyes immersion-fixed in 4% (w/v) paraformaldehyde (4 hours at room temperature). Eyes were cryoprotected by overnight immersion in 20% (w/v) sucrose solution at 4 °C. Eyes were then embedded in Optimal Cutting Temperature (O.C.T.) medium (R A Lamb, E. Sussex, UK) and frozen in isopentane that had been pre-cooled in liquid nitrogen. Specimens were stored at -80 °C. Sections were cut using a cryostat (Bright, UK) and kept frozen until use.

2.13.3 Evaluation of GFP fluorescence in cryosections

Sections were washed for 10 minutes in tap water to remove O.C.T. and counterstained using propidium iodide (Sigma-Aldrich Company Ltd.) at 1 μ g/ml in PBS (5 minutes). After washing (5 minutes in PBS) the sections were mounted in fluorescent mounting medium (Dako Ltd.) and a coverslip secured at its edges using nail varnish. Low power images were obtained using a Leica DMIL inverted microscope with a mercury vapour lamp and a GFP excitation filter. Higher power images were captured using a Zeiss LSM 510 confocal microscope operating a multi-tracking mode. GFP and propidium iodide were detected using a narrow banded FITC (488 nm) filter and a rhodamine/TRITC (543 nm) filter respectively.

2.13.4 Staining of sections by periodic acid and Schiff's reagent

Paraffin wax-embedded sections were washed in xylene and decreasing concentrations of alcohols in water. Sections were stained using periodic acid for 8 minutes and then washed 3 times in distilled water for 5 minutes. Sections were stained using Schiff's reagent for 8 minutes and then immersed in fast running tap water for 5 minutes to allow the pink colour to develop. Sections were counterstained by immersion in haematoxylin for 10 seconds. Slides were subsequently returned through increasing concentrations of alcohols to xylene and mounted in DPX.

2.13.5 Staining of sections by haematoxylin and eosin

Paraffin wax-embedded sections were washed in xylene and alcohols to water. Sections were stained using haematoxylin for 5 minutes and washed in distilled water. Sections were then stained using eosin for 5 minutes and washed in distilled water. Slides were subsequently returned through increasing concentrations of alcohols to xylene and mounted in DPX.

2.13.6 Immunostaining of vascular endothelial cells by anti-von Willebrand factor antibody.

Unfixed paraffin wax-embedded 6 μm sections were soaked in xylene to remove the wax, and then rehydrated in reducing concentrations of alcohols. Slides were then soaked in tap water for 10 minutes and PBS for 5 minutes. Slides were incubated for 30 minutes in blocking buffer (10% normal swine serum(Dako Ltd) with 10% normal mouse serum in 1% BSA/PBS) for 15 minutes. Slides were incubated for 30 minutes at room temperature with the primary antibody (Polyclonal rabbit anti-human von-Willebrand factor antibody (*Dako A0082*) at a dilution of 1:100. After three washes (PBS for 5 minutes) slides were incubated for 30 minutes with the secondary antibody, FITC-conjugated swine anti-rabbit antibody (Dako 0205) with 10% normal mouse serum in 1% BSA/PBS at a dilution of 1:30. The slides were washed again three times (0.1% BSA/PBS for 5 minutes). Counterstaining of the nuclei was performed using propidium iodide as described above and slides were mounted in fluorescent mounting

medium (Fluormount, Dako). Sections were examined by fluorescence microscopy and images captured using a digital camera.

2.13.7 Identification of cone photoreceptor cells in canine retina by peanut agglutinin staining

Cryosections were washed in tap water to remove the O.C.T., then left in TBS for 10 minutes. The sections were blocked for 30 minutes at room temperature in blocking buffer (TBS with 0.1 % (v/v) Tween 20, 3% (w/v) BSA and 10% (v/v) normal goat serum). Sections were incubated overnight at 4°C with biotinylated peanut agglutinin (Vector Laboratories, Burlingame, CA) at a concentration of 25 µg/ml in Tris-buffered saline with 0.1 % (v/v) Tween20 (TBS-T) and 0.3 % (w/v) albumin. After three washes with TBS-T the sections were incubated with Alexa 594-conjugated streptavidin (Molecular Probes Europe, Leiden, The Netherlands) in TBS-T for 2 hours at room temperature. Slide were washed twice (TBS with 0.1% (v/v) Tween 20 for 5 minutes) and then counterstained for 5 minutes using a 1 in 5000 dilution (in dH₂O) of DAPI (Sigma-Aldrich Company Ltd.). Slides were washed once (TBS for 5 minutes) and then mounted in fluorescent mounting medium (Dako Ltd.).

2.14 Buffers

Distilled water (dH₂O) was used to prepare all solutions. Sterile solutions were autoclaved at 121°C for 20 minutes unless otherwise stated.

Ampicillin (1000x) 50 mg/ml ampicillin (Sigma-Aldrich Company Ltd.) in dH₂O, sterile-filtered, stored at - 20 C.

Alkaline stock solution (10 x)

4 M NaOH and 100 mM EDTA.

Church buffer 21 g of NaH_2PO_4 , 48.55 g of Na_2HPO_4 , 70 g of SDS, 0.5 M EDTA and dH_2O to 1 litre.

DNA loading buffer (6x)

0.25% (w/v) bromophenol blue and 30% (v/v) glycerol in water.

Iodixanol (60%) solution

10 ml Opti-Prep™ 60% (w/v) iodixanol solution (Invitrogen, Ltd.) with 30 μl phenol red solution (0.5% - Invitrogen, Ltd).

LB (Luria-Bertani) bacterial growth medium

10 g Tryptone (Oxoid Ltd., Hampshire, UK), 5 g Yeast Extract (Oxoid Ltd.) and 10 g NaCl, water to 1 litre. 15 g/litre of Bacteriological Agar (No.1) (Oxoid Ltd.) added for LB agar plates.

PBS (1X) 85 g NaCl, 4.3 g KH_2PO_4 , 14.8 g Na_2HPO_4 , dH_2O to 10 litres, pH 7.2.

PBS (1X) for tissue culture

10 phosphate buffered saline tablets (Oxoid Ltd) dissolved in 1 litre ddH_2O , sterile.

PBS –MK 1 x PBS with 1 mM MgCl_2 and 2.5 mM KCl, sterile.

Proteinase K buffer 100 mM Tris (pH 7.4), 50 mM EDTA and 0.5% SDS.

Sodium cacodylate buffer

0.2 M $(\text{CH}_3)_2\text{AsO}_2\text{Na} \cdot 3\text{H}_2\text{O}$ with 0.2N HCl

Sodium phosphate buffer

97.1 g of Na_2HPO_4 , 43.6 g of NaH_2PO_4 , pH7.2 and dH_2O to 1 litre.

Storage buffer

20 mM Tris pH 8.0, 0.15 M NaCl and 2 mM MgCl_2 .

TAE (50X)

0.2 M Tris base, 1 M glacial acetic acid, 50 mM EDTA, pH 8.

TBS (1X)

85.3 g NaCl, 60.8 g Tris, 30 ml conc. HCl and dH_2O to 10 litres.

TE (pH 8.0)

10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

TMN

50 mM Tris pH 8.0, 5 mM MgCl_2 and 0.15 M NaCl, sterile

3

Angiostatic gene transfer in experimental retinal neovascularisation

This chapter describes the development and optimisation of a murine model of ischaemia-induced retinal neovascularisation, the characterisation in this model of gene expression mediated by adenovirus vectors and recombinant adeno-associated virus vectors, and the evaluation of the therapeutic and adverse effects of gene transfer of the soluble VEGF receptor sFlt-1.

3.1 Models of retinal neovascularisation

In order to determine the potential of an angiostatic gene transfer approach to retinal neovascularisation the first step was to identify and establish an appropriate experimental model. A number of models of retinal neovascularisation have been described. Diabetes can be experimentally induced by a galactose-rich diet or by administration of streptozotocin. Diabetic animals develop diabetes-like histopathological retinal changes including basement membrane thickening, pericyte degeneration, capillary dilation, microaneurysm formation and endothelial cell proliferation but, unless also exposed to 1500 cGy of radiation,³¹⁰ tend not to develop clinically significant retinal neovascularisation. The spontaneously diabetic Torii (STD) rat typically develops proliferative diabetic retinopathy at 70 weeks of age with tractional retinal detachment followed by intraocular hemorrhage.³¹¹ The late development of retinal

neovascularisation in this model, however, is impractical for the purpose of this study for which and a model of more rapidly developing retinal neovascularisation is required.

Pre-retinal neovascularisation can be induced by experimental retinal vein occlusion. Laser-induced retinal vein occlusion, with or without photodynamic thrombosis, induces local tissue hypoxia and vasoproliferative microangiopathy in pigs^{312,313} and rats.^{314,315} Laser vein occlusion in primates results in retinal ischaemia and iris neovascularisation⁷³ but fails to induce retinal neovascularisation.⁶⁴

The identification of VEGF as a critical mediator of angiogenesis has led to the development of animal models of retinal neovascularisation induced by VEGF. These include intravitreal sustained release devices in rabbits³¹⁶ and VEGF overexpression in transgenic mice. Since anti-VEGF approaches are attractive therapeutic strategies, however, choosing to evaluate these in a model induced directly by VEGF might be considered limited.

The clinical association of retinopathy of prematurity with exposure to hyperoxia has led to the development of rodent models of oxygen-induced retinal neovascularisation. Exposure of newborn rats to 80 % oxygen, alternating with short episodes in room air for 11 days, followed by continuous exposure to room air for 6 days consistently results in peripheral retinal ischaemia and secondary neovascularisation.³¹⁷ Pre-retinal neovascularisation can also be induced in mouse pups by exposure to high levels of oxygen from postnatal day 7 (p7) to p12.³⁰⁸ Retinal vasculature development in mice is almost complete by p7. Exposure to hyperoxia at this age results in extensive retinal capillary closure at the posterior pole around the optic disc. This pattern of retinal ischaemia and neovascularisation contrasts with the pattern seen in the rat model and in human ROP in which ischaemia and neovascularisation occur in the peripheral retina. The capillary oblitative response in the mouse model is associated with down-regulation of retinal VEGF expression³¹⁸ and may result from failure of VEGF-dependent survival of the immature vasculature through selective apoptosis of endothelial cells.³¹⁹ Although the molecular mechanisms are not well understood, nitric oxide is believed to

play an important role; the vaso-obliterative effects of hyperoxia are significantly reduced in mice without functional endothelial nitric oxide synthase (eNOS) or in mice given pharmacological inhibitors of eNOS. On subsequent return to room air, the relative hypoxia in the areas of ischaemic retina results in upregulation of VEGF expression by ganglion cells¹¹⁹ and VEGF-dependent⁵³ pre-retinal neovascularisation in 100% of animals. Continued exposure to sustained hyperoxia results in the accelerated recovery of inner retinal vasculature and prevents pre-retinal vascularisation by a mechanism that may involve the preservation of microglia.³²⁰ Retinal capillary sensitivity to hyperoxia may be a unique feature of early retinal vascular development and not a feature of newly forming capillaries in general. Although the mouse model of oxygen-induced retinal neovascularisation does not exactly mimic human disease and is less similar to ROP than the rat model, it is a reliable model of ischaemia-induced retinal neovascularisation in general. This murine model is well characterised, highly reproducible, rapidly induced and widely accepted.

3.2 Murine model of ischaemia-induced retinal neovascularisation

Having determined that the murine model of ischaemia-induced retinal neovascularisation was appropriate for the investigation of angiostatic gene transfer, this model was established in the laboratory; the induction of retinal neovascularisation was demonstrated by several methods and the optimal method of quantification was determined.

3.2.1 Induction of ischaemia and retinal neovascularisation

All animals were treated in a humane manner and were managed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Female Pregnant female C57Bl/6J mice were purchased from a commercial source (Harlan, UK). Ischaemic retinopathy was induced in mouse pups by exposure of to $75 \pm 2\%$ oxygen

from postnatal day p7 to p12 in a custom-built chamber (Figure 3.1) along with their nursing dams. Room temperature was maintained at 20° C and illumination provided by standard fluorescent lighting on a 12-hour light–dark cycle. Pups were nursed by their dams and given food (standard mouse chow) *ad libitum*. Litters of control mice were housed in room air continuously.

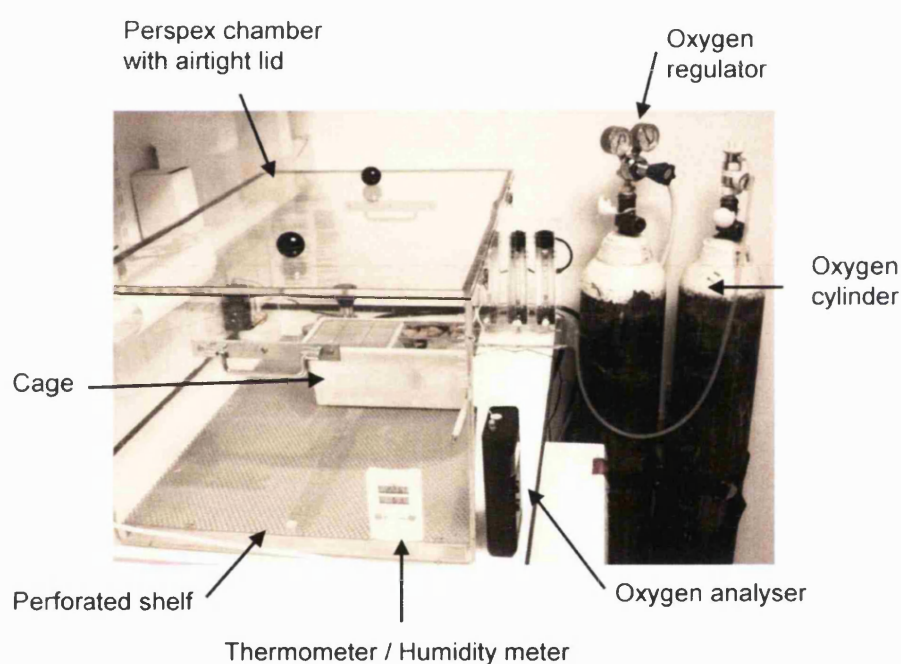


Figure 3.1 Custom-built perspex chamber for controlled exposure of mouse pups to hyperoxia.

Oxygen is pumped into the chamber at a flow rate such that its concentration is maintained at approximately 75%.

3.2.2 Analysis of retinal neovascularisation

In order to demonstrate induction of retinal neovascularisation in animals exposed to hyperoxia and to determine optimal methods of quantification, experimental and control animals were examined by *in-vivo* fluorescein angiography and their retinae analysed by fluorescein-dextran perfusion and histological staining techniques.

3.2.2.1 In-vivo fundus fluorescein angiography

Fundus fluorescein angiography was performed at P19 in animals exposed to hyperoxia and in normal age-matched controls raised in room air continuously. Briefly, mice were anaesthetised and their pupils dilated before intraperitoneal injection of sodium fluorescein. A series of angiograms were acquired during a 10 minute period using a Kiowa Genesis small animal fundus camera with appropriate filters.

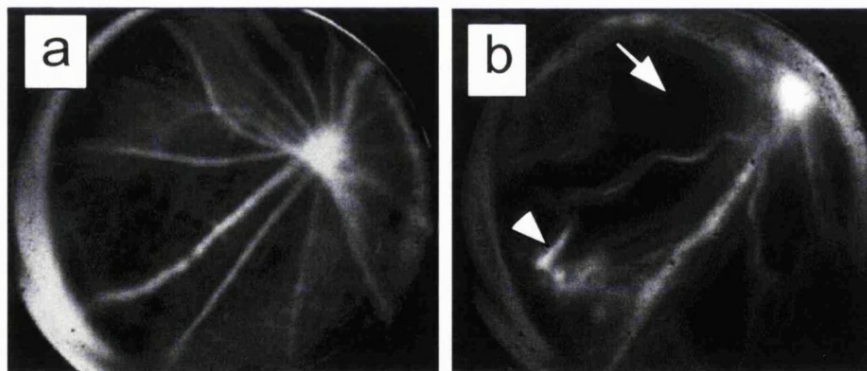


Figure 3.2 Representative *in-vivo* fundus fluorescein angiograms of animals at p19, 120 seconds after intraperitoneal injection of sodium fluorescein.

a, In an animal raised in room air continuously retinal vasculature is normal; b, In an animal after exposure to hyperoxia followed by return to room air there are extensive areas of hypofluorescence at the posterior pole of the retina (white arrow) consistent with capillary non-perfusion and an area of intense hyperfluorescence at the border of the perfused and non-perfused retina (white arrowhead) consistent with fluorescein leakage from a retinal neovascular complex.

In control mice at p19 raised in room air continuously, fluorescein angiography demonstrates a normal pattern of retinal vasculature with fully perfused capillary network and no significant vascular leakage (Figure 3.2). In contrast, in experimental mice at p19 exposed to 75% oxygen from p7 to p12 followed by return to room air fluorescein angiography is strikingly abnormal. There are extensive areas of hypofluorescence at the posterior pole of the retina resulting from capillary non-perfusion and areas of intense hyperfluorescence at the border of the perfused and non-perfused retina consistent with retinal neovascularisation.

3.2.2.2 Fluorescein-dextran perfused fused whole retina mounts

At p19 fluorescein-dextran perfused fused whole retina mounts were prepared from experimental and control animals. Animals were terminally perfused using fluorescein-labelled dextran and their retinas flat-mounted for examination by fluorescence microscopy. Its conjugation with high molecular weight dextran maintains fluorescein within the retinal vasculature such that it tends not to leak even from immature neovascular complexes. In retinas from control animals at p19 raised in room air continuously, fluorescein-dextran perfused retinal flatmounts clearly demonstrate a normal pattern of retinal vasculature with a fully perfused capillary network (Figure 3.3). In contrast, in experimental animals at p19 exposed to hyperoxia followed by normoxia, the vascular pattern in fluorescein-dextran perfused retinal flatmounts is strikingly abnormal; there are extensive areas of hypofluorescence at the posterior pole near the optic nerve head consistent with capillary non-perfusion and areas of intense hyperfluorescence at the border of the perfused and non-perfused retina consistent with complexes of retinal neovascularisation.

3.2.2.3 Histological examination of retinal neovascularisation

Control and experimental animals were sacrificed at p19, their eyes enucleated, fixed and embedded in paraffin wax. Sections were stained using periodic acid and Schiff's reagent to delineate the inner limiting membrane of the retina and haematoxylin to

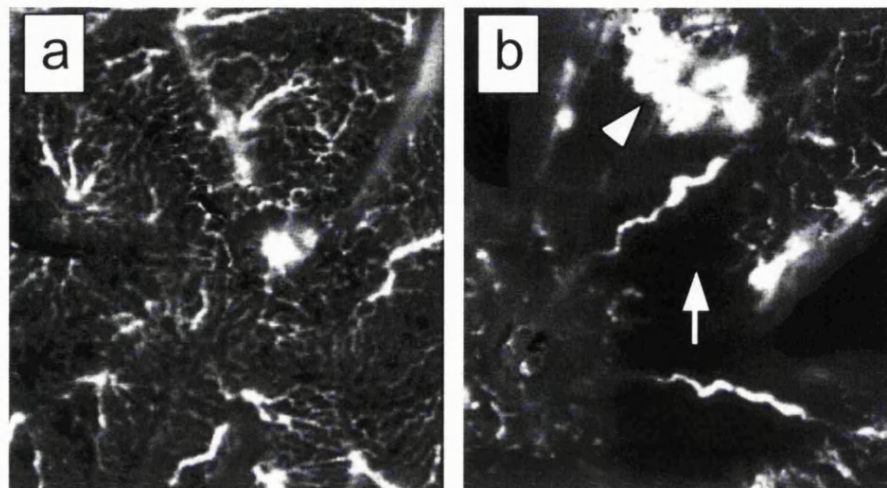


Figure 3.3 Representative fluorescein dextran-perfused retinal flatmounts of animals at p19.

a, In an animal raised in room air continuously retinal vasculature is normal; b, In an animal after exposure to hyperoxia followed by return to room air there are extensive areas of hypofluorescence at the posterior pole of the retina (white arrow) consistent with capillary non-perfusion and an area of intense hyperfluorescence at the border of the perfused and non-perfused retina (white arrowhead) consistent a retinal neovascular complex.

identify cell nuclei. The retinal architecture of mice at p19, raised continuously in room air, appeared entirely normal with a smoothly defined inner limiting membrane and no cell nuclei present on the vitreous side of the inner limiting membrane (Figure 3.4a). In retinal sections from mice at p19 exposed to 75 % oxygen from p7 to p19 there is evidence of abundant cellular proliferation anterior to the inner limiting membrane consistent pre-retinal neovascularisation (Figure 3.4b).

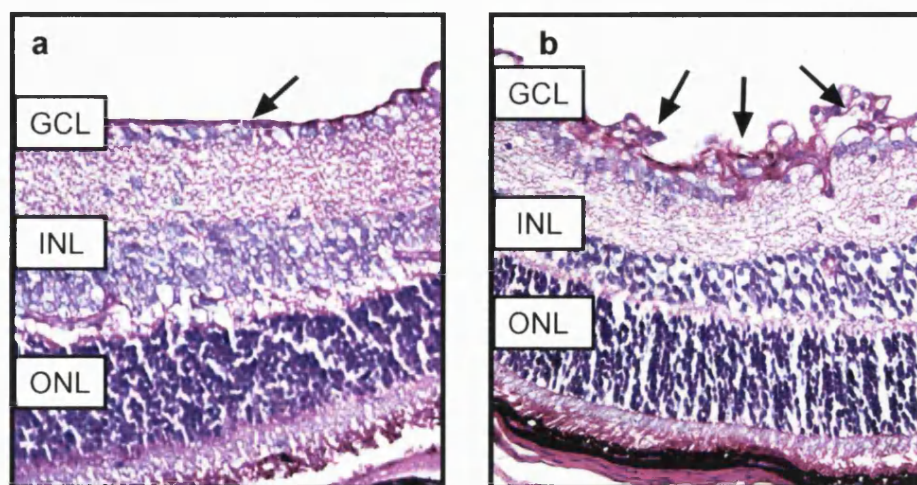


Figure 3.4 Histological sections of retinas mice at p19

a, In retinal section from animal raised continuously in room air the retinal architecture is normal with no cell nuclei present on the vitreous side of the inner limiting membrane of the retina (black arrow); *b*, In section from animal following exposure to hyperoxia and return to room air there is evidence of abundant cellular proliferation anterior to the inner limiting membrane (black arrows). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

3.2.2.4 Quantification of pre-retinal neovascularisation

Four 6 μm sagittal sections, each at least 50 μm apart, on each side of optic nerve were stained with periodic acid, Schiff's reagent and haematoxylin. The number of neovascular endothelial cell nuclei on the vitreous side of the inner limiting membrane of the retina in each section was counted at x400 magnification using a masked protocol. The mean number of cell nuclei per section per eye was used as a single experimental value.

In retinas from control animals at p19 (n=6) raised in room air continuously, the mean number (SD) of cell nuclei on the vitreal aspect of the inner limiting membrane of the retina was $0.5 (\pm 0.8)$. In contrast, in animals p19 exposed to hyperoxia followed by normoxia (n=6) the mean number (SD) of cell nuclei on the vitreal aspect of the inner limiting membrane was 49.9 ± 10.8 ($p < 0.0001$) (Figure 3.5).

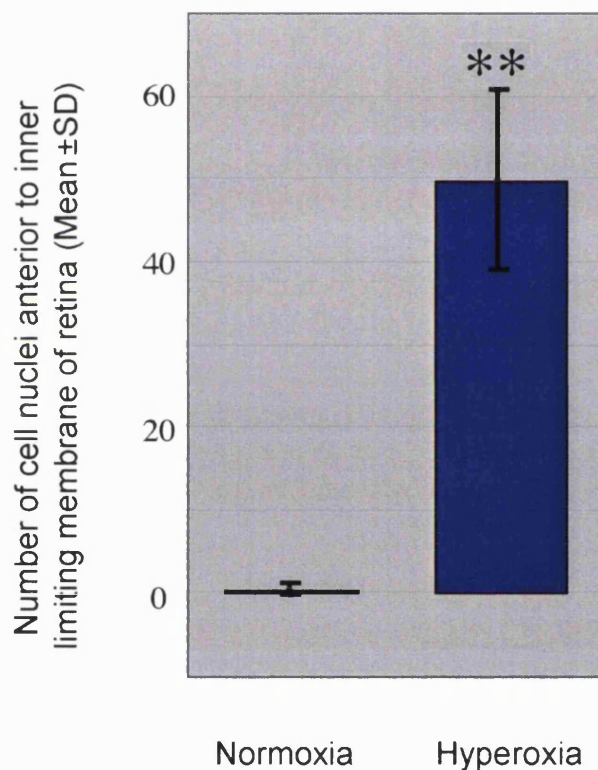


Figure 3.5 Quantification of retinal neovascularisation in murine ischaemia-induced retinopathy

Retinal neovascularisation was quantified at p19 in animals exposed to room air continuously (normoxia) and in animals exposed to 75% oxygen from p7 to p12 (hyperoxia) (** $p < 0.0001$).

3.3 Reporter gene expression by adenovirus and adeno-associated virus vectors in murine model of retinal neovascularisation

Having established and optimised a model of retinal neovascularisation, the next objective was to achieve effective local tissue expression angiostatic proteins by gene transfer. Two different viral vector systems, Ad and AAV vectors, were evaluated because while they are both known to mediate efficient transduction of intraocular tissues the two vectors have contrasting patterns of tissue transduction and contrasting time-courses of transgene expression. After intravitreal injection in normal adult mice the transduction efficiencies and tissue specificities of both Ad³²¹ and AAV vectors²⁷⁰ are well described. In murine ischaemic retinopathy, however, the young age of the animals and their exposure to hyperoxia are factors that might potentially influence the vector tropisms and the kinetics of transgene expression. In order to determine the pattern of transgene expression following intra-ocular administration of the vectors in this particular model, either adenovirus or AAV vectors encoding the reporter gene green fluorescent protein (GFP) driven by a CMV promoter were introduced into the vitreous (Figure 3.6).

Ad and AAV vectors mediate contrasting time-courses of transgene expression. Previous studies have indicated that Ad-mediated expression begins within 24 hours and is short-lived; immune responses to the vector gene products limit the duration of expression to 3 weeks in the eye.²¹³ In contrast, AAV-mediated expression is delayed, maximal expression is normally established only after 6 weeks in the retina^{243,270} and expression is subsequently sustained for months or years.³²² In the mouse model of retinal ischaemia, retinal neovascularisation is short-lived, reaching a maximum level at p17-p21 and subsequently regressing spontaneously.³⁰⁸ The timing of vector delivery in this experiment was chosen in an attempt to coordinate optimal vector-mediated

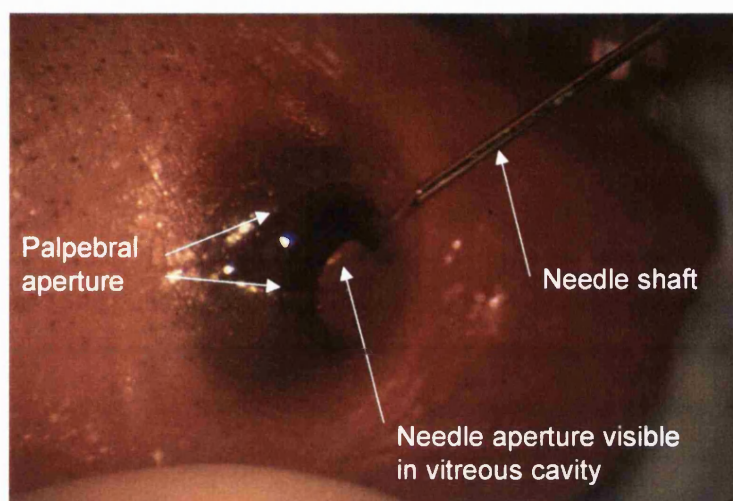


Figure 3.6 Intravitreal delivery of viral vector

The palpebral fissure was opened and the pupil dilated. The anterior part of the vitreous was visualised using an operating microscope. The tip of a 10 mm 34-gauge needle, mounted on a 10 μ l Hamilton syringe, was advanced under direct vision through the sclera, 1 mm posterior to the corneoscleral limbus, into the vitreous and a volume of approximately 1 μ l of viral suspension was injected into the vitreous cavity.

expression with the peak of anigogenic drive, which occurs shortly before the peak of neovascularisation at approximately p15.⁵³ Since the two vectors have different time-courses of expression different injection time-points were chosen for each. The Ad vector was injected at p12 because we anticipated expression to be maximal within a few days but rapidly attenuated thereafter, whereas the AAV vector was injected at the earliest opportunity, p2, to compensate for an anticipated delay in transgene expression. The eyes were enucleated and analysed for vector-mediated expression at p16, 4 days after the onset of ischaemia-induced retinal neovascularisation. Cryosections were counterstained with propidium iodide and examined by fluorescence microscopy.

3.3.1 Adenovirus-mediated expression in ischaemia-induced retinopathy

No GFP fluorescence was observed in the anterior segment or the retina of uninjected eyes (n=4) (Figure 3.7a). Following intravitreal delivery of the Ad vector (n=6) GFP reporter gene expression was evident in cells of the corneal endothelium, iris pigment epithelium and ciliary epithelium (Figure 3.7b) but not in the retina (except for a very small number of cells in the inner nuclear layer). There was no GFP fluorescence evident in cells of the pre-retinal neovascular complexes.

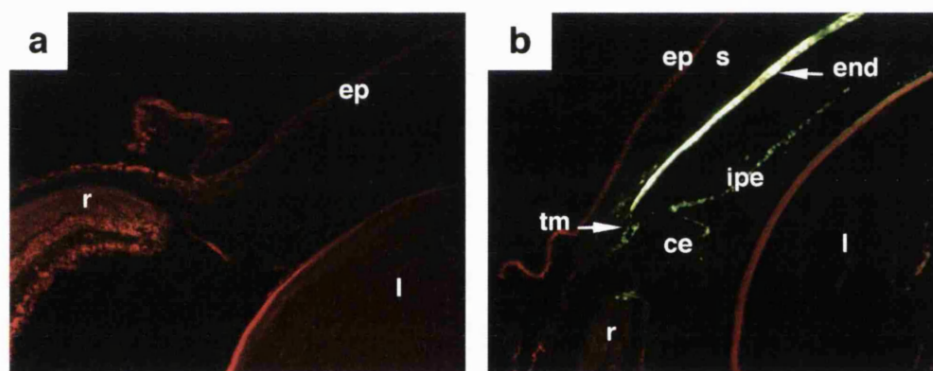


Figure 3.7 Ad-mediated reporter gene expression following intravitreal injection
Fluorescence micrographs showing GFP expression in the anterior segment of eyes following intravitreal vector delivery in murine ischaemia-induced retinopathy. a, in the anterior segment of an uninjected control eye no GFP fluorescence is evident; b, in the anterior segment following intravitreal delivery of Ad vector, GFP fluorescence is evident in corneal endothelium (end), trabecular meshwork (tm) iris pigment epithelium (ipe) and ciliary epithelium (ce). GFP expression is not evident in corneal epithelium (ep), corneal stroma (s) or retina (r).

3.3.2 AAV-mediated expression in ischaemia-induced retinopathy

Following intravitreal delivery of the AAV-2 vector (n=6) GFP reporter gene expression was evident in retinal cells across a large area of the posterior pole (Figure 3.8b).

Expression was most prominent in ganglion cells and in cells of the inner nuclear layer, but was also seen in a number of cells in the outer nuclear layer. There was no evidence of gene transfer to ciliary epithelial or corneal endothelial cells by AAV-2 and no GFP expression in cells of the pre-retinal neovascular complexes.

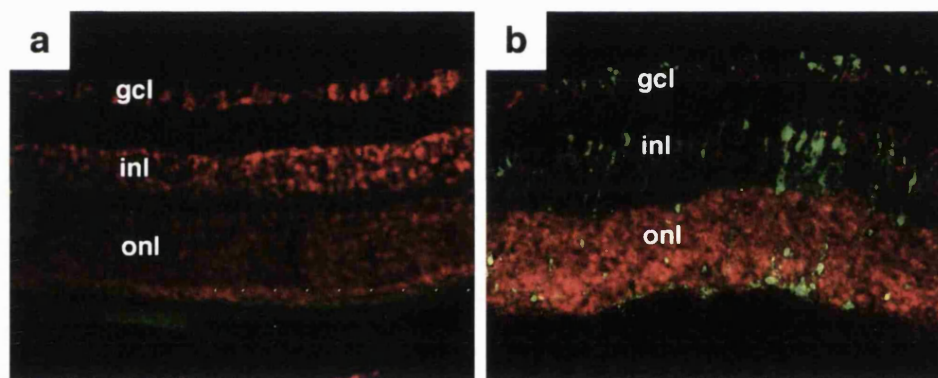


Figure 3.8 AAV-mediated reporter gene expression following intravitreal injection
Fluorescence micrographs showing AAV-mediated GFP expression in the retinae of eyes following intravitreal vector delivery in murine ischaemia-induced retinopathy. a, uninjected control eye, no GFP fluorescence is evident; b, following intravitreal delivery of AAV vector, GFP fluorescence is evident primarily in ganglion cell layer (gcl) and inner nuclear layer (inl). GFP positive photoreceptor cells are also present in the outer nuclear layer (onl).

3.3.3 AAV-mediated expression following intravitreal delivery in young normal mice

AAV-mediated transduction of photoreceptors normally requires vector delivery to the subretinal space. The observation in this model that a significant number of photoreceptors can be transduced by an AAV vector delivered into the vitreous has important implications for clinical applications of retinal gene transfer. Possible reasons that may explain this unexpected pattern of transgene expression include the young age (p2) at which the animals were injected and their subsequent exposure to 75% oxygen. In order to determine whether AAV-mediated photoreceptor transduction following intravitreal delivery is age-dependent, a further experiment was designed to investigate the pattern and timecourse of transgene expression after intravitreal injection of normal (non-ischaemic) mice at different ages.

Intravitreal injections of AAV.CMV.GFP were performed in eyes of mice at p2 (n=6), p7 (n=6), or p14 (n=6). The procedure was performed as previously described. All animals were raised in room air continuously and were not exposed to hyperoxia. Mice were sacrificed by cervical dislocation 2, 4 and 8 weeks after vector delivery. Retinal cryosections were counterstained with propidium iodide and examined by confocal microscopy for expression of GFP.

GFP was efficiently expressed in cells of the ganglion cell layer in all animals, regardless of the timing of vector delivery, though the onset of expression was more rapid in younger animals than in older animals (Figure 3.9). Following vector delivery at p7 or p14, GFP expression was confined largely to the ganglion cell layer, consistent with previous reports. In contrast, vector delivery at p2 resulted in significant GFP expression observed in cells of the inner nuclear layer and the outer nuclear layer (Figure 3.9b, 3.9c). The efficiency of photoreceptor transduction in these animals, determined by calculating the proportion of cells in the outer nuclear layer that expressed GFP, was estimated to be 3 % to 5 %.

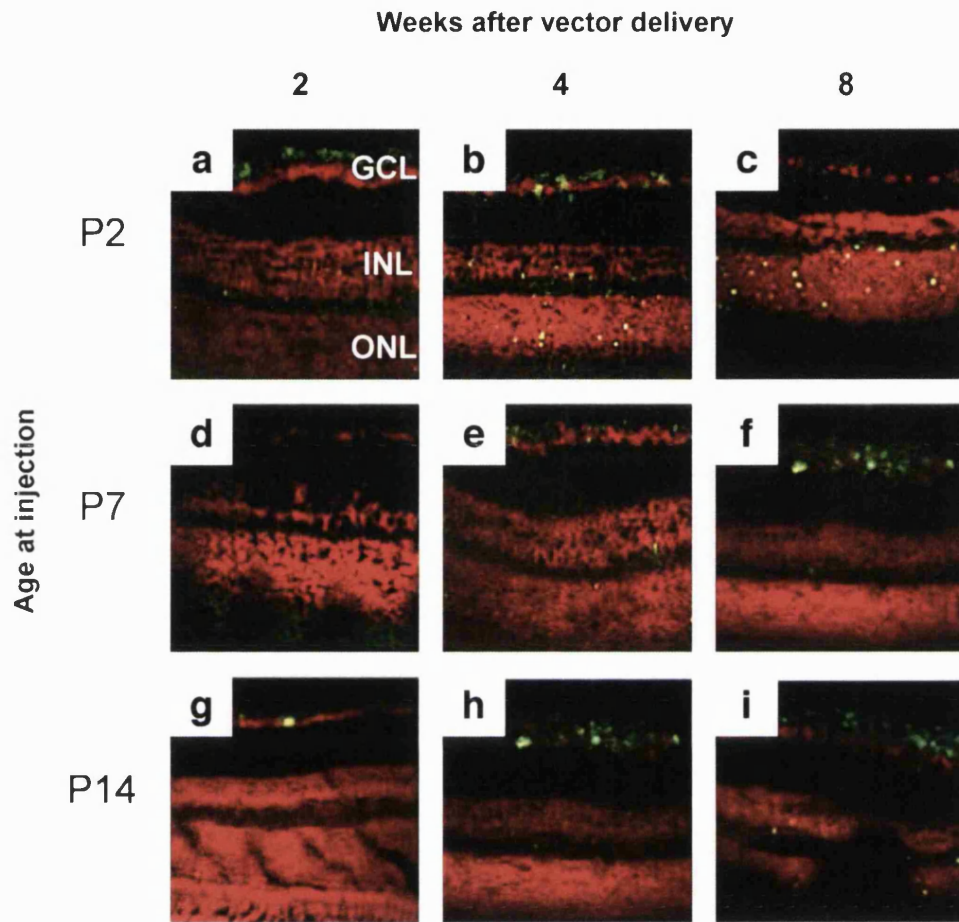


Figure 3.9 AAV-mediated expression following intravitreal vector delivery in normal mice at different postnatal ages

Representative confocal fluorescence micrographs; vector was delivered at p2 (a, b, c), p7 (d, e, f) or p14 (g, h, i). Analysis was performed 2 weeks (a,d,g) 4 weeks (b, e, h) or 8 weeks (c, f, i) following vector delivery. GFP fluorescence in photoreceptor cells in the outer nuclear layer is most marked following intravitreal vector delivery at p2, evident at 4 weeks (b) and at 8 weeks (c) following vector delivery. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

3.4 Gene transfer of soluble VEGF receptor sFlt-1 by adenovirus and AAV vectors in murine model of retinal neovascularisation

Having established the murine model of retinal ischaemia and demonstrated efficient reporter gene expression following intravitreal delivery of Ad and AAV vectors, both these vector systems were used to deliver genes encoding angiostatic proteins to the retina with the aim of reducing the extent of retinal neovascularisation. In view of the extensive evidence for the central role of VEGF in retinal neovascularisation, the effect of VEGF inhibition by gene transfer of the soluble VEGF receptor sFlt-1 was investigated.

3.4.1 Construction and production of rAAV vector expressing the soluble VEGF receptor sFlt-1

3.4.1.1 Cloning of AAV.CMV.*sFlt-1*

A recombinant AAV plasmid vector containing the *sFlt-1* cDNA driven by a CMV promoter and flanked at the 3' end with a SV40 polyadenylation signal was constructed. The cDNA encoding the sFlt-1 was amplified by PCR from a recombinant adenovirus vector encoding the sFlt-1 DNA (a kind gift from Dr R.Crystal, Cornell University, New York, NY) using the forward primer (TCGGGATCCTCGCCACCATGGTCAGCTACTGGGACACC) that incorporated a *Bam*HI site and a reverse primer (ATAGCGGCCGCTTAATGTTTACATTACTTTGTGTGGT) that incorporated a *Not*I site. The PCR was carried out using the proof reading enzyme *Pfu* polymerase (Stratagene, Amsterdam, Holland) (Figure 3.10).

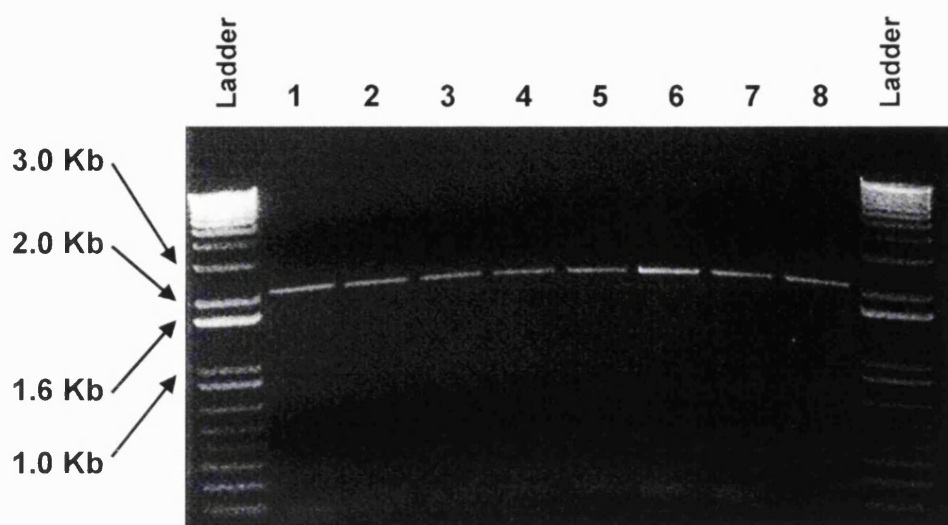


Figure 3.10 PCR of *sFlt-1* cDNA from *Ad.CMV.sFlt-1* construct.

The expected 2.1 Kb product is evident in all 8 sample lanes.

The 2.1 kb PCR product was digested with *Bam*HI and *Not* I then sub-cloned into the mammalian expression cassette pEGFP-N1 (Clontech, Basingstoke, UK) vector by substituting the *Bam*HI-*Not* I EGFP cDNA fragment with the PCR amplified fragment to yield *psFlt-1-NI*. Successful cloning was confirmed by a restriction enzyme digest using *Bam*HI and *Not* I of 6 minipreps (Figure 3.11).

The *sFlt-1* cDNA was verified by sequencing (Lark Technologies, Saffron Walden, UK). A 1.4 Kb fragment of the yeast (*s.cerevisiae*) gene histidinol dehydrogenase (*HIS4*) was amplified by PCR from pPIC9 plasmid (Invitrogen) for use as a stuffer fragment using the forward primer (5' TCG ACA TGT ACC GGT GCC ATT TGC CTG 3') that incorporates a *Afl* III site and the reverse primer (GCT ATT AAT TGG TCC AGG AAA CCA GTT) that incorporates a *Ase* I site. The *psFlt-1-NI* plasmid was digested with *Ase* I and *Afl* III and the *HIS4* stuffer fragment was inserted into this to yield *psFlt-1-NI-stuffer*. The *psFlt-1-NI-stuffer* was digested with *Age* I and *Nae* I, and derivative of AAV plasmid psub201³²³ that was deleted for all viral genes was digested using *Age* I and *Eco*R V. The resulting fragments were ligated to produce the plasmid pAAV.CMV.*sFlt-1*.

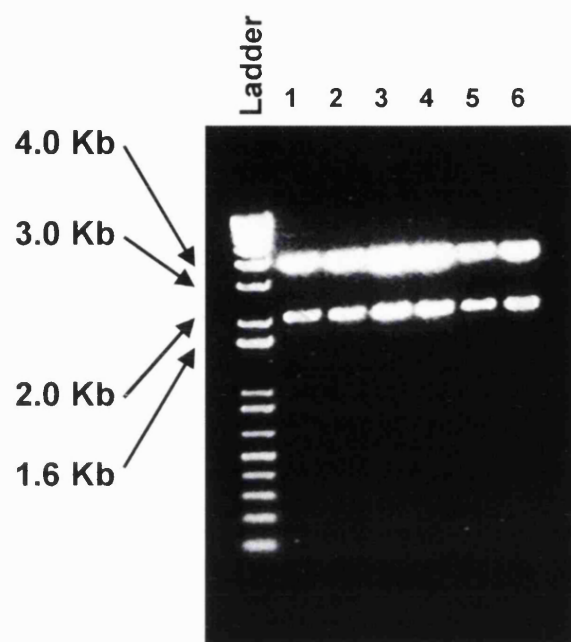


Figure 3.11 Restriction enzyme digest of *psFlt-1-NI* by *Bam*HI and *Not* I
2.1kb *Flt* fragment and 4.0 kb vector backbone fragment are evident in all of
6 minipreps.

3.4.1.2 Production of AAV.CMV.*sFlt-1* vector

Recombinant AAV-2 plasmid expressing GFP driven by the CMV promoter (pHAV 5.5) was produced using the replicating amplicon system described previously²²⁸ and purified using sodium deoxycholate followed by a one step affinity purification procedure on a heparin column.²²⁷ Titration of vector preparation was performed by dot blot using a CMV probe.

3.4.1.3 Analysis of sFlt-1 expression by AAV.CMV.sFlt-1 *in vitro* by Western blot

In order to demonstrate expression of sFlt-1 protein by the AAV vector *in vitro*, Western analysis (Figure 3.12) was performed on the conditioned medium from transduced 293 cells, using a sFlt-1-specific antibody (1:500 goat R&D Systems, Abingdon, UK) as described in Chapter 2. OptiMEM and the conditioned media from transduced by rAAV.CMV.PEDF were used as negative controls.

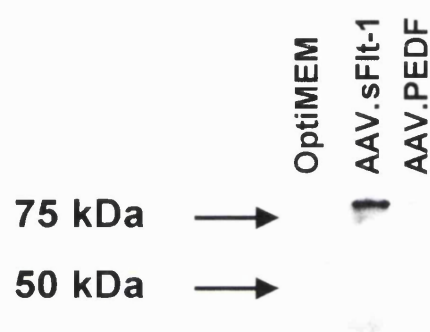


Figure 3.12 Western blot confirming production of sFlt-1 by 293T cells following infection by AAV.CMV.sFlt-1
A band showing protein of the expected size (77.5kDa) is evident in the AAV.sFlt-1 lane.

3.4.2 Determination of adenovirus-mediated and AAV-mediated *in-vivo* expression of sFlt-1

In-vivo expression of sFlt-1 following intravitreal delivery of Ad and rAAV vectors was quantified by ELISA of pooled homogenised eyes. The Ad.CMV.sFlt-1⁷⁶ virus was a kind gift of Marc Feldman. Retinal ischaemia was induced in newborn mice by exposure to 75% oxygen from p7 to p12 as described previously. Intravitreal delivery of rAAV.CMV.sFlt-1 was performed in animals at p2 and delivery of Ad.CMV.sFlt-1 at p12. Animals were sacrificed at p15 by cervical dislocation and their eyes snap-frozen

un-fixed in liquid nitrogen. Attempts to localise sFlt-1 by immuno-histochemical techniques were unsuccessful due to technical difficulties of localising the soluble antigen. Eyes were pooled and manually ground in assay buffer with proteinase inhibitors. Following centrifugation at 4000 rpm for 5 minutes the supernatant was assayed for total sFlt-1 using a total sFlt-1 test kit (RELIATech, Braunschweig, Germany) as described in Chapter 2.

In eyes injected with Ad.CMV.sFlt-1 ($n=6$), sFlt-1 was detected at a concentration of 72 ng/ml. In eyes injected with rAAV.CMV.sFlt-1 ($n=4$), sFlt-1 was detected at a concentration of 0.1 ng/ml. sFlt-1 was undetectable in pooled uninjected eyes ($n=6$).

3.4.3 Evaluation of angiostatic effect of Ad-mediated and AAV-mediated sFlt-1 expression in experimental retinal neovascularisation

Having confirmed in-vivo local expression of sFlt-1 mediated by both Ad and AAV vectors, the effect of this expression on the extent of retinal neovascularisation was evaluated qualitatively and quantitatively and the possibility of adverse effects were investigated in normal animals. Retinal ischaemia was induced in newborn mice by exposure to 75% oxygen from p7 to p12 as described previously. Mice underwent intravitreal delivery of AAV.CMV.sFlt-1 at p2 or Ad.CMV.sFlt-1 at p12. Fluorescein-dextran perfused whole retina mounts were obtained at p19 for qualitative evaluation of retinal neovascularisation. Assessment of retinal flatmounts indicated fewer neovascular complexes in eyes injected with rAAV.CMV.sFlt-1 and Ad.CMV.sFlt-1 than in uninjected eyes or in eyes injected with PBS, Ad.CMV.GFP or AAV.CMV.GFP (Figure 3.13).

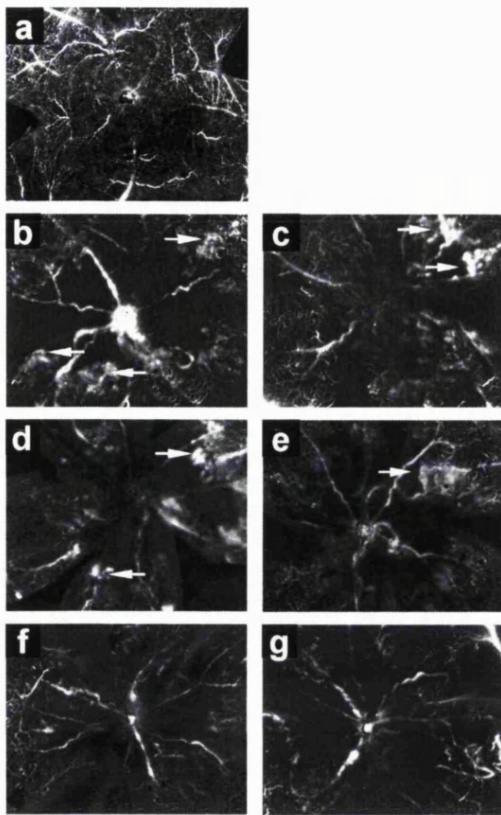


Figure 3.13 Representative FITC-perfused retinal flatmounts at p19

Retinal neovascularisation appears as areas of hyperfluorescence and is indicated (white arrows). (a) Normal age-matched non-ischaemic control. No areas of hyperfluorescence are evident. (b-g) retinas of hyperoxia-exposed mice following different treatment interventions; (b) uninjected; (c) injected with PBS; (d) Ad.CMV.GFP; (e) AAV.CMV.GFP; (f) Ad.CMV.sFlt-1 and (g) AAV.CMV.sFlt-1.

The extent of neovascularisation was quantified by counting the number of endothelial cell nuclei anterior to the inner limiting membrane of the retina in serial sections at p19. Statistical analysis was performed using ANOVA and Student's t-test with Bonferroni's correction for multiple comparisons. The mean (\pm SEM) was 48.2 (4.1) in uninjected eyes (n=7), 42.1 (5.0) in eyes injected with PBS (n=7), 34.8 (3.3) in eyes injected with Ad.CMV.GFP (n=9), 21.4 (4.3) in eyes injected with Ad.CMV.Flt-1 (n=7) ($P < 0.05$), 52.1 (6.9) in eyes injected with AAV.CMV.GFP (n=7), and 23.3 (6.6) in eye injected with AAV.CMV.sFlt-1 (n=6) ($p < 0.05$) indicating a significant reduction in neovascularisation following local delivery of a gene encoding sFlt-1 (Figure 3.14).

sFlt-1?

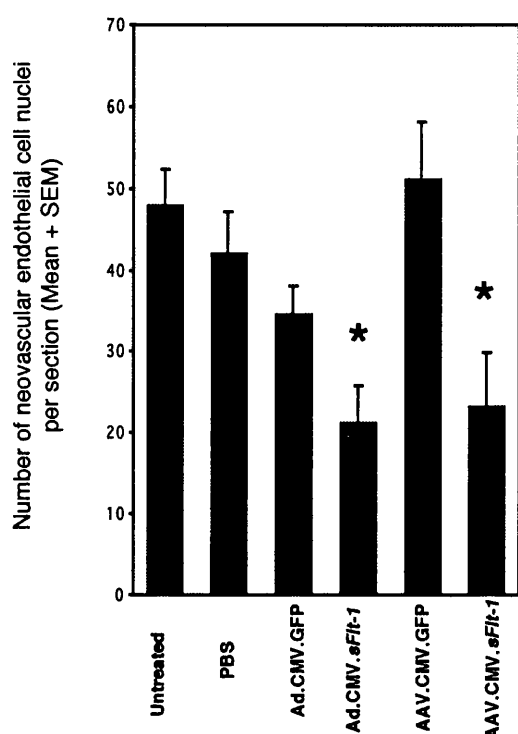


Figure 3.14 Quantification of angiostatic effect of local sFlt-1 gene transfer on experimental retinal neovascularisation

The mean number of neovascular cell nuclei at p19 in eyes injected with Ad.CMV.sFlt-1 or AAV.CMV.sFlt-1 was approximately 50% of the number in uninjected eyes (* $p < 0.05$) in both cases. Differences between uninjected eyes and eyes injected with PBS or viruses expressing GFP were not statistically significant.

3.4.4 Evaluation of adverse effects of Ad-mediated and rAAV-mediated sFlt-1 expression in normal animals

In order to determine any potential adverse effects of viral gene transfer of sFlt-1 on normal retinal vascular development or architecture vectors expressing sFlt-1 were injected into the vitreous of normal (non-ischaemic) animals. Ad.CMV.sFlt-1 was injected at p12 and rAAV.CMV.sFlt-1 at p2. All animals were sacrificed at p19. Fluorescein-dextran perfused retinal flatmounts were prepared to investigate any effect on normal vascular development. In the uninjected eye of a normal mouse the vessels form a fine radial branching pattern in the superficial layer and a polygonal reticular pattern in the deep layer (Figure 3.15-1). This vascular pattern is preserved after intravitreal injection of Ad.CMV.sFlt-1 at p12 (Figure 3.15-2) or injection of

AAV.CMV.sFlt-1 at p2 (Figure 3.15-3) and there is no evidence of altered vascular permeability.

To investigate possible adverse effects on retinal architecture 6 μ m H&E stained retinal sections of normal (non-ischaemic) mice injected in the same way were examined by light microscopy (Figure 3.15d,h,i). All layers of the retina appeared normal and we observed no evidence of an inflammatory cell infiltrate or retinal thickening.

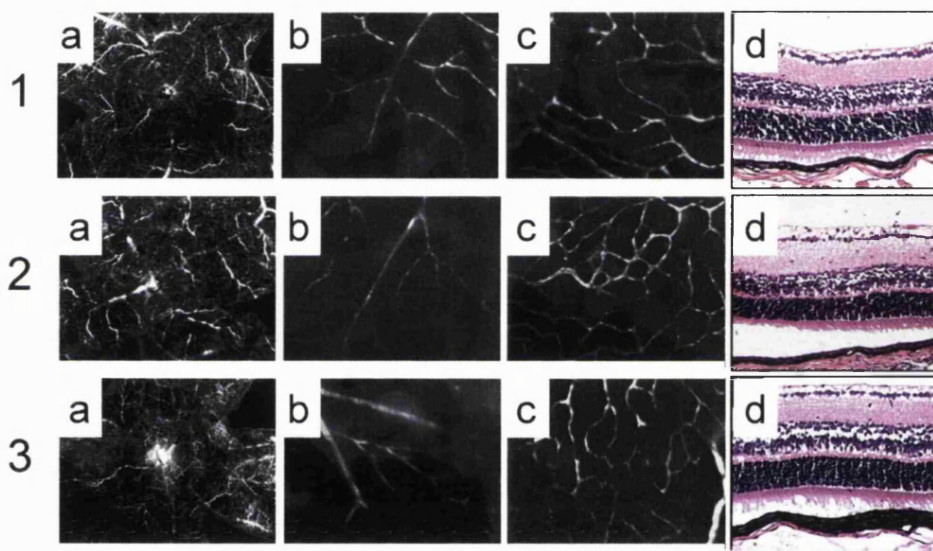


Figure 3.15 Determination of potential toxicity resulting from gene transfer of sFlt-1 in normal (non-ischaemic) mice

1, Uninjected controls; 2, Injected with Ad.CMV.sFlt-1; 3, Injected with AAV.CMV.sFlt-1. (a), FITC-dextran perfused retinal flatmounts (40X objective). (b) Detail of superficial (inner retinal) vascular network at high magnification (40X objective). (c) Detail of deep (outer-retinal) vascular network at high magnification (40X objective). The appearance of the retinal vascular networks is similar in all treatment groups. (d) Light micrographs of H&E- stained sections. The retinal architecture appears normal with no evidence of inflammatory cell infiltration or retinal thickening.

3.5 Discussion

3.5.1 Murine model of ischaemia-induced retinal neovascularisation

A variety of techniques usefully demonstrate the dramatic pre-retinal neovascularisation that occurs in this model as a result of exposure to hyperoxia followed by room air. The appearances of dextran-fluorescein perfused retinal flatmounts in experimental and control animals are consistent with those of *in vivo* angiograms. Retinal flatmounts offer greater ease of imaging with higher magnification resulting in higher resolution images and provide superior definition of the vasculature. This offers a useful qualitative means to assess retinal neovascularisation in this model. Since dextran-fluorescein does not leak from new vessels, however, the identification of new vessels in flat-mounted retinas was dependent on vessel morphology as opposed to hyperfluorescence and the discrimination of neovascular complexes from normal vasculature on this basis was not always clear. Methods to quantify neovascularisation in flatmounts by counting the number of clock hours in which neovascular complexes are apparent have been described, as have counting the total number of complexes. However, in view of the difficulties in confidently identifying neovascular complexes using this method, and the subjective interpretation that this implies, perfused flatmounts were used only for illustrative purposes in this study and they were not used to quantify the extent of neovascularisation.

In contrast, the counting of nuclei on the vitreous surface of the retina offered an objective and reproducible means to quantify retinal neovascularisation. Neovascular quantification has been described by immuno-staining of endothelial cell in serial sections followed by automated image analysis. This technique is highly labour-intensive, however, and does not appear to offer a significant advantage over conventional staining. For these reasons PAS/Haematoxylin staining of serial sections was selected as the most reliable and feasible method for the accurate quantification of retinal neovascularisation in this model. This model has proven to be reliable, reproducible and quantifiable,

yielding useful results within a reasonably short time-scale. A degree of variation in neovascular score between eyes is apparent but this can be minimised by optimising study design, specifically through the inclusion of appropriate controls and the use of an untreated contra-lateral eye as an internal control for each treated eye.

3.5.2 Reporter gene expression by Ad and AAV vectors in murine model of retinal neovascularisation

The purpose of these experiments was to characterise the transfection efficiency and tissue specificity of Ad and AAV vector systems in a murine model of ischaemia-induced retinal neovascularisation. In order to control pre-retinal neovascularisation the aim was to achieve effective concentrations of transgene product in the area of retinal ischaemia. The intravitreal route of vector delivery was chosen because this route is most likely to result in transgene expression in the inner retina. The tissue tropisms of the vectors after intravitreal delivery in adult mice have been well described^{270,286,324} but any differences specific to delivery in young mice or in the context of retinal ischaemia have not previously been evaluated.

The results presented in this thesis confirmed that the vectors have contrasting tissue specificity of transfection in this model just as they do in adult mice without retinal ischaemia. Despite being delivered into the vitreous, Ad-mediated transgene expression was confined almost exclusively to cells in the anterior segment, including the corneal endothelium, the trabecular meshwork and the ciliary epithelium. GFP expression was also observed in a very small number of cells in the inner nuclear layer consistent with transfection of occasional Müller cells. Transduction of retinal Müller cells has previously been reported after intravitreal injection of adenoviral vectors but its efficiency is relatively low unless delivery is combined with a vitrectomy,²⁷¹ a procedure not performed in the present study. Overall the pattern and efficiency of adenovirus-mediated transfection in this murine model of ischaemic retinopathy is similar to that observed in normal adult mice.

Intravitreal injection of AAV in adult mice normally results in transduction of ganglion cells exclusively. AAV-mediated expression in mouse pups after exposure to hyperoxia, however, was also present in a proportion of cells in the outer and inner nuclear layer in addition to ganglion cells. Although co-localisation using immuno-staining techniques would be required to definitively demonstrate that the transduced cells are photoreceptors, their appearance and location are entirely consistent with this. Efficient transfection of photoreceptor cells by AAV normally necessitates vector delivery to the subretinal space, a procedure that is technically more demanding and potentially more damaging to the retina than is intravitreal injection, particularly in very young animals. In order to determine whether the unusual transduction pattern observed in this experiment is a feature of young animals or whether it is related to the induction of retinal ischaemia, a further experiment was designed to investigate the transfection pattern after intravitreal delivery of AAV in normal (non-ischaemic) mice at a range of postnatal ages. The results of this study demonstrated that significant photoreceptor transfection was achieved in normal animals after neonatal (p2) injection regardless of exposure to hyperoxia and induction of ischaemia, and the young age of the animals is sufficient to explain AAV-mediated photoreceptor transfection after intravitreal delivery. Age-dependent photoreceptor transduction following intravitreal delivery of AAV may be explained by improved access of viral particles to photoreceptors in an immature developing retina. The ability to target photoreceptor cells by intravitreal vector delivery, at least in very young rodents, may be valuable for further experimental studies. This finding does not necessarily apply to humans, however, since, in contrast to rodents, human retinal development is relatively complete at birth.

In order to target retinal neovascularisation effectively, an ideal vector would lead to effective concentrations of the expressed protein in areas of retinal ischaemia. Where the therapeutic protein is secreted and soluble, such as sFlt-1 or PEDF, it would not seem essential to achieve transduction of cells directly involved in the angiogenic process. Indeed delivery of neither Ad nor AAV vectors results in transduction of cells in the new vessel complexes themselves, presumably because neither vector has a natural tropism for vascular endothelial cells and because the vectors were introduced some days in

advance of the onset of endothelial cell proliferation. Efficient expression by any local cell population might result in effective concentrations of angiostatic molecules.

Assuming, however, that local expression will result in a concentration gradient of the therapeutic protein, transduction of cells in close proximity to areas of retinal ischaemia would be expected to be more effective than transduction of cells at a distance. For this reason the tropism of AAV for cells of the inner retina seems preferable for targeting angiogenic processes in areas of retinal ischaemia than the Ad vector that transduces predominantly cells some distance away in the anterior segment of the eye.

Since the two vector systems have different time-courses of expression, different injection time-points were used accordingly. The Ad vector was injected at p12 because adenovirus-mediated expression was expected to be maximal within a few days but rapidly attenuated thereafter. Analysis of GFP fluorescence confirmed efficient adenovirus-mediated reporter gene expression 3 days after intravitreal delivery. The delay between AAV delivery and onset of transgene expression presented a potential disadvantage for the utility of this vector system in this particular model. It was not possible to induce experimental neovascularisation in older animals in the same way because older mice are resistant to hyperoxia-induced retinopathy.³⁰⁸ Delivery of AAV vector, however, at the earliest opportunity in neonatal animals did result in significant gene expression by p15. This level of expression is likely to represent only a fraction of the maximal expression that would be expected after a further 4 weeks.²⁴³

3.5.3 Gene transfer of soluble VEGF receptor sFlt-1 by adenovirus and rAAV vectors in murine model of retinal neovascularisation

Local gene transfer and expression of sFlt-1 by both Ad and AAV vectors inhibited experimental retinal neovascularisation by 56% and 52% respectively. At present, it is unclear whether residual neovascularisation is due to incomplete inhibition of the VEGF response, or rather from the uninhibited activity of an alternative angiogenic pathway.

The ability of a VEGF receptor kinase inhibitor to prevent neovascularisation completely in this model suggests that improved gene delivery and expression might be expected to overcome this limitation.⁵² It remains to be seen whether the angiostatic effect can also be enhanced by gene transfer of dual or multiple angiostatic factors.

The findings of the present study are consistent with previous studies of VEGF inhibition in which recombinant proteins have been delivered by repeated intravitreal injection in this model of ischaemia-induced retinopathy. Retinal neovascularisation is reduced by 47% after repeated injection of recombinant sFlt-1⁷³ and by 31% after repeated injections of VEGF antisense oligodeoxynucleotides.⁷⁴ In a separate study the author has shown that a seven amino acid residue peptide RKRKKS (EG3306) from exon 6 of VEGF that inhibits VEGF receptor binding and angiogenesis *in vitro* also results in a 51% inhibition of retinal neovascularisation following repeated intravitreal injection *in vivo*.³²⁵ Though repeated intravitreal injections of small molecules such as these can be effective in this model of short-lived neovascularisation, this approach is not likely to be feasible in longer-term neovascular disorders in which a more sustained anti-angiogenic effect is required.

Neovascularisation in the murine model of ischaemia-induced retinopathy is self-limiting; after p21 no further proliferation occurs and the new vessels regress spontaneously.³⁰⁸ Even in this model in which the neovascular response is relatively transient, delivery of sFlt-1 by gene transfer after a single procedure is as effective as repeated intravitreal injections of recombinant sFlt-1 or the 7mer from VEGF exon 6.³²⁵ The advantage of a gene transfer approach in facilitating sustained inhibition is likely to be of greater importance when applied to conditions in which there is a longer-term predilection to neovascularisation. Although AAV-mediated expression of sFlt-1 is likely to be sustained, the long-term effect on neovascularisation cannot be confirmed in this model and will need to be evaluated in further appropriate models and clinical studies.

In this study the efficacies of two different viral vector systems with contrasting expression profiles were evaluated. Although more rapid in onset of expression,

adenoviral vectors are less attractive than AAV vectors for clinical application because of their immunogenicity, toxicity, and short-lived expression.³²⁶ The two vectors also have contrasting spatial patterns of gene transfer and transgene expression. Intravitreal injection of Ad vectors results in transduction of the corneal endothelial cells, iris, ciliary body and trabecular meshwork.^{286,324} Although transduction of retinal Müller cells has previously been reported after intravitreal injection of Ad vectors^{212,321,327} this is inefficient unless the injection is combined with a vitrectomy.²⁷¹ Although attempts to demonstrate tissue localisation of sFlt-1 by immuno-histochemistry were unsuccessful due to technical difficulties of locating soluble antigens, it is speculated that a substantial proportion of soluble protein secreted by cells in the anterior segment and ciliary body may be directed by aqueous flow anteriorly through the pupil towards the trabecular meshwork. This pattern of expression may be particularly well suited to the delivery of an angiostatic molecule in the management of neovascularisation of the iris and irido-corneal angle. However, the efficacy of Ad vectors in reducing retinal neovascularisation also suggests that sFlt-1 diffuses posteriorly from the anterior segment across the vitreous body to the inner retina in quantities sufficient to achieve significant VEGF inhibition. In contrast, intravitreal injection of AAV almost exclusively results in reporter gene expression by cells in the retinal ganglion cell layer and inner nuclear layer, consistent with transduction of ganglion cells and Müller cells. The proximity of these cells to the developing neovascular complexes in the retina may explain a comparable angiostatic effect despite relatively low levels of sFlt-1 expression. The localisation of gene expression close to the site of pathology, and the known biological characteristics of gene transfer by AAV vectors makes this a highly attractive strategy for sustained therapy of retinal neovascularisation.

VEGF is implicated in retinal vascular development and acts as a survival factor for retinal vascular endothelium.³¹⁹ For this reason possible effects of sFlt-1-mediated VEGF inhibition on retinal vascular development and architecture were investigated in the normal mouse. The development of the retinal vasculature in mice occurs during the first two weeks of life.³²⁸ From p0 to p10 vessels develop radially by vasculogenesis from the optic disc to the ora serrata to form a superficial vascular network within the nerve fibre

layer of the inner retina. From p4 vessels extend from these superficial vessels by angiogenesis towards the outer retina leading to the formation of a deep vascular plexus. The results described in this thesis demonstrate that both superficial and deep retinal vascular networks appear normal despite sFlt-1 expression by either vector system. We also found the retinal architecture to be unaffected. Although we observed no evidence of an inflammatory cell infiltrate in H&E-stained histological sections, an immune response to the Ad vector is well-described in the literature and is usually only apparent after immuno-phenotyping.²¹³ Whilst these results suggest that sFlt-1-mediated VEGF inhibition has no dramatic adverse effect on retinal vascular pattern or morphology, there remains the possibility that sustained, complete or uncontrolled inhibition of VEGF may cause undesirable effects on endothelial cell homeostasis. Incorporation of regulatable expression elements in the vector will minimise these possibilities, and such a strategy is described in Chapter 5.

Acknowledgements

I gratefully acknowledge the advice and assistance of Ajay Mistry in the cloning and production of AAV.CMV.*sFlt-1*. The Ad.CMV.*sFlt-1* virus was a kind gift of Marc Feldman and the Ad.CMV.GFP virus was provided by Keith Channon.

4

Angiostatic gene transfer in experimental choroidal neovascularisation

This chapter describes the development of a reproducible model of choroidal neovascularisation, the characterisation of gene expression mediated by rAAV and lentiviral vectors, and the evaluation of the therapeutic and adverse effects of gene transfer of the soluble VEGF receptor sFlt-1 in this model.

4.1 Development of a model of choroidal neovascularisation

The development of a valid model of choroidal neovascularisation requires an understanding of its pathogenesis in humans. Briefly, age-related maculopathy is characterised by abnormalities of the extracellular matrix at the level of Bruch's membrane that present a diffusion barrier to water-soluble constituents of plasma.¹³ These abnormalities are associated with changes in the retinal pigment epithelium including atrophy and the induction of a pro-angiogenic phenotype.¹⁷ Neovascularisation originates from the choroidal vasculature and extends into the sub-RPE space through defects in Bruch's membrane. This process occurs in association with the local upregulation of pro-angiogenic cytokines including VEGF in particular.^{69,329} The mechanisms leading to the

induction of VEGF expression in this condition are not well understood but one possibility may involve hypoxia of overlying RPE cells^{17,18} resulting from thickening of Bruch's membrane or abnormalities of choroidal perfusion.¹⁴⁻¹⁶ Choroidal neovascularisation can occur as a secondary manifestation of a number of conditions other than AMD. These conditions include angioid streaks, high myopia, trauma, choroidal tumors, familial macular dystrophies, and inflammatory retinochoroidopathies,^{330,331} and are similarly characterised by the presence of breaks in Bruch's membrane that permit the growth of choroidal new vessels into the subretinal space. Although these conditions are relatively uncommon they may be at least as amenable to angiostatic approaches and have the advantage that they tend not to be associated with atrophy of RPE.

There is no experimental animal model that reproduces all the features of age-related macular degeneration. Although intra-retinal or intra-choroidal angiogenesis can be induced by the local overexpression of VEGF, the neovascular complex fails to extend across an intact Bruch's membrane.^{71,309,332} In contrast, laser-induced rupture of Bruch's membrane is sufficient alone to induce upregulation of VEGF expression^{333 334,335} and the development of a neovascular complex that extends from the choroidal vasculature into the subretinal space. First described in primates,³³⁶ models of laser-induced choroidal neovascularisation are now extensively used in rabbits,³³⁷ rats,^{338,339 334,340} and mice^{341,342 343} to identify angiogenic proteins, determine the role of specific genes with knockout mice, and evaluate the efficacy and safety of anti-angiogenic therapies. Lasers with a range of different wavelengths have been employed including krypton red,^{341,342} diode³⁴⁰, argon³⁴⁴ and dye lasers.⁷⁵ The laser injury is typically applied using a slitlamp-mounted delivery system with power settings ranging from 100 mW to 400 mW, pulse duration from 50 msec to 100 msec and spot size from 50 μ m to 100 μ m in diameter, typically applying 4 to 8 burns at the posterior pole of each eye. The induced neovascular response can be evaluated and quantified by a number of means. *In vivo* fluorescein angiography demonstrates an area of diffuse hyperfluorescence at the site of laser injury reflecting perfusion, leakage and staining of the neovascular membrane.³³⁸ Fluorescence microscopy of flatmounts of the RPE/choroid/sclera following terminal perfusion with

FITC-dextran demonstrate the intravascular component of the new vessel complex.^{307,344}

Quantification of angiographic neovascularisation has been described in terms of the incidence of neovascular lesions induced per burn,^{341,345} the area of fluorescence per burn^{346,347} and by scoring systems to grade the ‘activity’ or fluorescein leakage from the induced lesion. An alternative to fluorescein angiography is the immunohistochemical staining of vascular endothelial cells in histological sections of the induced lesion to enable quantification of the area of neovascular endothelium per lesion.^{342,343}

On the basis that laser-induced choroidal neovascularisation reflects the upregulation of VEGF expression and defects in Bruch’s membrane that characterise the human disorder, a model of laser-induced choroidal neovascularisation in mice was established and refined in order to optimise its reproducibility with a view to the evaluation of angiostatic intervention.

4.1.1 Application of laser injury

A slit-lamp mounted diode laser system was first assembled. A table-mounted slit-lamp (Keeler UK) was fitted with a diode laser of wavelength 680 nm (Keeler UK). The laser delivery system was mounted directly in line with the slit-lamp illumination and viewing system and operated by means of a footswitch. An aluminium platform designed for the purpose provided a stable support to maintain the animal in the horizontal plane at the level of the laser-delivery system. Adult C57Bl/6J mice were anaesthetised by intraperitoneal injection of Hypnorm and Hypnovel in distilled water and pupillary mydriasis induced using topical atropine 1%. The anaesthetised animal was positioned on the aluminium platform such that its eye was brought directly into alignment with the axis of the laser (Figure 4.1). A plano contact lens system, comprising a cover-slip and hydroxypropylmethycellulose contact solution, was applied to the eye and the fundus brought into focus. Four laser burns were applied to the posterior pole of the retina approximately 3 disc diameters from the optic disc, avoiding major retinal vessels. Laser

settings of 100 mW, 100 ms duration, 100 μ m diameter were used. These settings resulted consistently in an immediate chalk-white blanching of the retina with variable formation of a small subretinal air bubble and occasional minor choroidal haemorrhage (Figure 4.2).

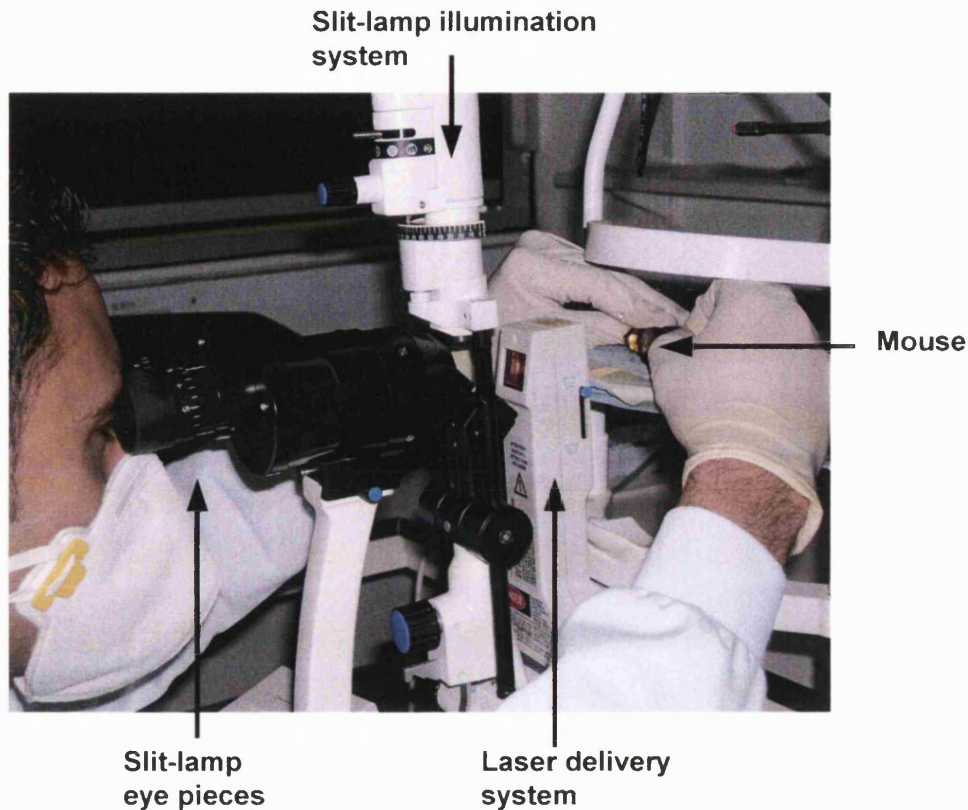


Figure 4.1 *Diode laser induction of choroidal neovascularisation using a slit-lamp mounted delivery system*

The mouse is supported on a steel platform and orientated to bring its eye into alignment with the aiming beam.

As an alternative to the slit-lamp mounted laser, an indirect ophthalmoscopy delivery system was also evaluated. A 78 diopetre converging lens was used in conjunction with a head-mounted indirect laser ophthalmoscope. The mouse fundus was brought into focus using the converging lens and 4 laser burns applied to the posterior pole of the retina using similar settings to those described above. This system resulted in chalk-white blanching of the retina in the majority of burns but achieved less consistent immediate

effects than the slitlamp-mounted alternative. This inconsistency is believed to be the result of the inability to standardise the spot size except through the subjective impression of the aiming beam diameter by the operator.

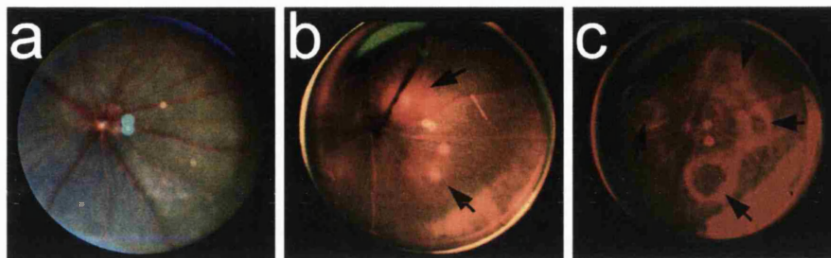


Figure 4.2 *Mouse fundus photographs showing effect of diode laser injury*

a, normal fundus prior to laser-injury; b, immediately following laser application; gas bubbles are formed subretinally at the sites of laser injury (arrows); c, 2 weeks later pigmentary disturbance is evident at the sites of laser injury and subretinal fibrovascular proliferations extends from these sites, in this example coalescing with those of adjacent lesions.

4.1.2 *In vivo* fluorescein angiography

Fundus fluorescein angiography was performed 14 days following laser rupture. The techniques developed for mouse fundus photography and fundus fluorescein angiography are described fully in Chapter 2. Briefly, animals underwent intraperitoneal anaesthesia and pupillary mydriasis. A Kowa Genesis small animal fundus camera was used in fluorescence angiography mode to employ the appropriate integrated excitation and emission filters. Sodium fluorescein 2 % was administered by intraperitoneal injection at a dose of 0.2 ml per 30 g body weight. Ilford 1600 ASA black and white film was exposed at intervals following fluorescein administration and push-processed at 3200 ASA to obtain angiograms of optimal contrast. Retinal vessels typically began to fill with fluorescein after 45 seconds and capillary washout occurred approximately 7-8 minutes

after dye administration. Areas of hyperfluorescence, evident at an early stage and increasing in area and brightness over time were evident at sites of laser-rupture consistent with choroidal neovascularisation (Figure 4.3).

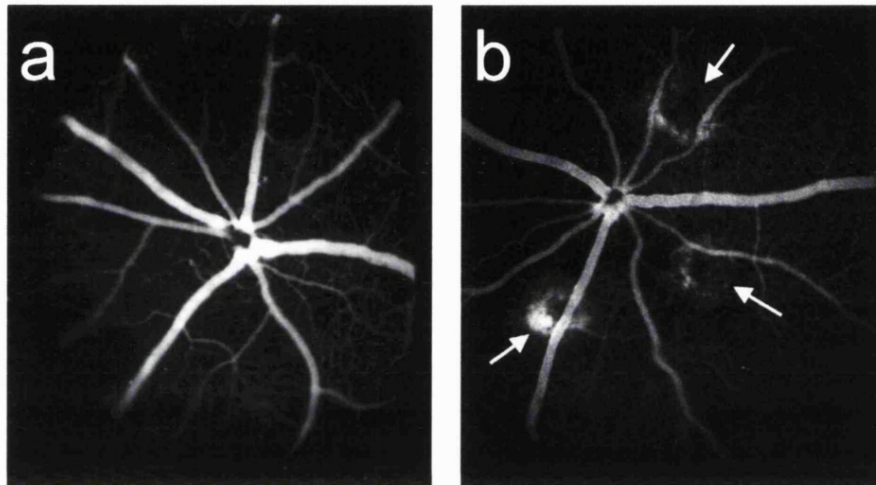


Figure 4.3 *In vivo fundus fluorescein angiograms showing effect of laser injury*
a, Normal mouse fundus fluorescein angiogram; *b*, 2 weeks following laser-induced rupture of Bruch's membrane areas of hyperfluorescence at sites of laser injury (arrows) are consistent with choroidal neovascularisation.

4.1.3 FITC-dextran perfused choroidal flatmounts

In order to investigate a potential alternative method of evaluation, FITC-dextran perfused retinal flatmounts were also prepared. Briefly, intra-cardiac perfusion of deeply anaesthetised animals was performed using FITC-labelled dextran. Flatmounts of the sclera/choroid/Bruch's/RPE complex were prepared from fixed eyes and examined by fluorescence microscopy. Since dextran does not leak even from newly forming vessels, this technique results in clear demonstration of the intravascular compartment of the

neovascular complex. Fluorescence microscopy demonstrated networks of vessels at the sites of laser membranotomy that were qualitatively different in structure to the pattern of the underlying choroidal vasculature (Figure 4.4).

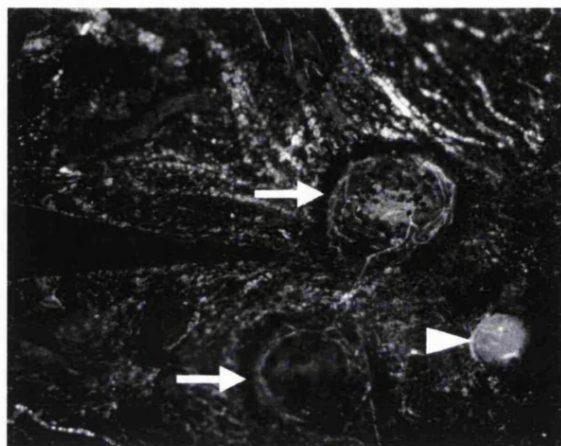


Figure 4.4 Fluorescence microscopy of FITC-dextran perfused choroidal flatmounts following laser-induced rupture of Bruch's membrane

Networks of vessels are evident (arrows) at sites of laser injury. The optic nerve head is indicated by an arrowhead.

4.1.4 Histological evaluation of laser-induced choroidal neovascularisation

In order to investigate the histological features of the laser-induced lesion two weeks following laser-injury, eyes were sectioned for conventional staining and for immunohistochemical staining (Figure 4.5). Haematoxylin and eosin staining demonstrated the presence of a fibro-cellular mass between the neurosensory retina and Bruch's membrane that was associated with focal disruption of the retinal pigment epithelium and of photoreceptor cell outer segments (Figure 4.5b). Immunohistochemical staining for vascular endothelial cells using anti-von Willbrand's factor antibody clearly

demonstrated the presence of positively staining between the choroidal vasculature and the outer retinal plexus (Figure 4.5d) that is not evident in a control eye that did not undergo laser injury (Figure 4.5c).

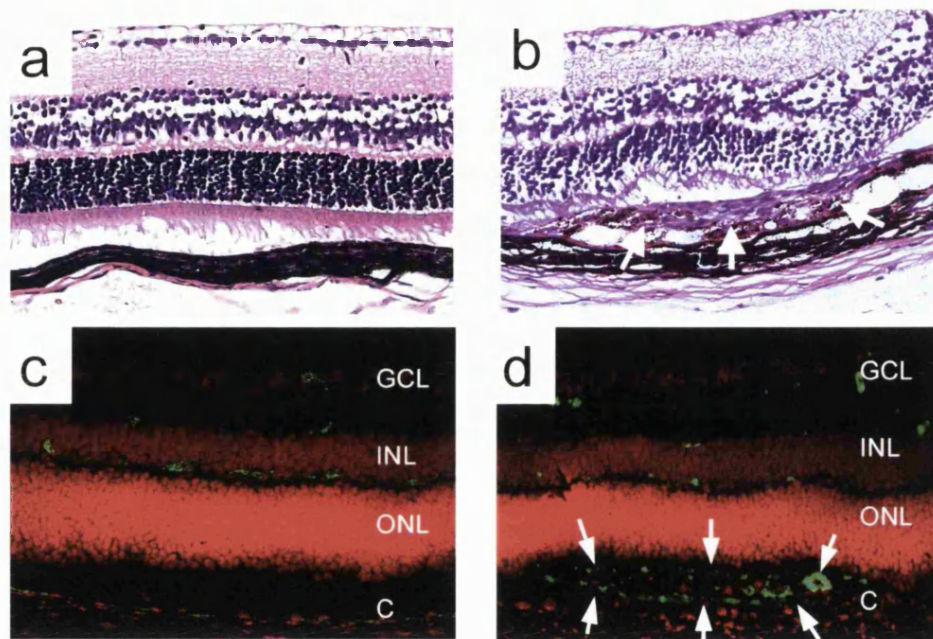


Figure 4.5 Histological analysis of effect of laser-injury

a, H&E-stained section of normal retina; b, H&E-stained section at site of laser injury 2 weeks following application of laser. A cellular proliferation is evident in the subretinal space (arrows); c, Immuno-staining of vascular endothelial cells by anti-von Willebrand factor antibody in normal retina. Positive staining (green) is evident in the inner retina, corresponding to the superficial and deep retinal capillary networks, and in the choroid (c) d, Immuno-staining of vascular endothelial cells at site of laser injury 2 weeks following application of laser. Extensive staining of endothelial cells evident in the subretinal space (arrows) demonstrates a significant vascular component to the tissue response to laser injury.

4.1.5 Image quantification of choroidal neovascularisation

In vivo fluorescein angiography was selected as the primary technique for evaluation of the extent of laser-induced choroidal neovascularisation for this project since it reflects a clinically relevant outcome and accounts for extravascular leakage, an indication of the activity of the neovascular process. Although FITC-dextran perfused flatmounts clearly demonstrate the intravascular component of the induced complex, these tissue specimens are time-consuming to prepare and give no indication of the activity of the lesion in terms of extravascular dye leakage. Histological delineation of induced choroidal neovascularisation (in contrast to pre-retinal neovascularisation) requires immunohistochemical identification of vascular endothelial cells in order to differentiate them apart from neighbouring tissues. The immunohistochemical analysis of serial sections in large numbers of eyes is labour intensive and was not predicted to offer significant additional information.

The extent of the laser-induced neovascular lesions was quantified by image analysis of *in vivo* fundus fluorescein angiograms. Exposures acquired 3 minutes following intraperitoneal fluorescein administration were selected for quantification since at this time point fluorescein leakage from the lesion was well established but after 3 minutes image quality tended to deteriorate as a result of fluorescein leakage into the vitreous. Images were digitalised and the areas of neovascularisation were measured using image analysis software (Image Pro Plus, Media Cybernetics, Silver Spring, MD, USA) (Figure 4.6). Either the area of hyperfluorescence resulting from each laser lesions laser lesion or the sum of hyperfluorescent areas per eye were used as a single experimental value.

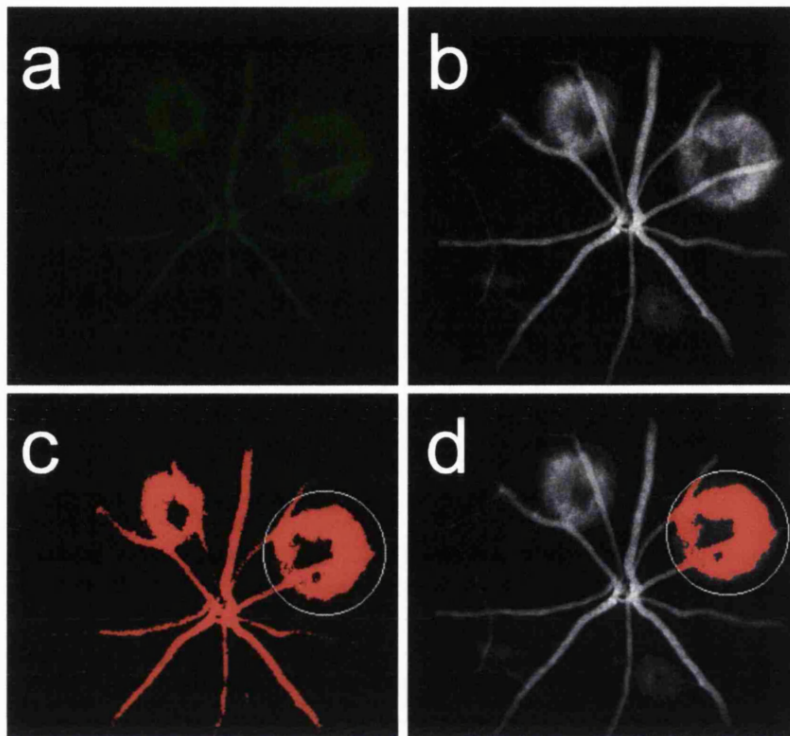


Figure 4.6 Image quantification of area of hyperfluorescence at sites of laser injury on in-vivo fluorescein angiography

Colour images (4.6 a) were converted to grey-scale format (4.6b) and areas of hyperfluorescence at sites of laser injury were selected as areas of interest by their manual delineation from surrounding areas (4.6 c). The area of the hyperfluorescent lesion within each area of interest (4.6c) was calculated and described in terms of the number of bright pixels. Either the area of hyperfluorescence resulting from each laser lesions laser lesion or the sum of hyperfluorescent areas per eye were used as a single experimental value.

4.1.6 Reproducibility of laser-induced choroidal neovascularisation

A wide range of species, laser types and laser configurations has been described in laser-induced models of choroidal neovascularisation. In order to optimise the reproducibility of the proposed mouse model for the subsequent evaluation of potential angiostatic therapies, the effects of different diode laser power and spot-size settings on both the incidence and the extent of the induced lesion were investigated. Mice underwent laser-injury as described above using a slit-lamp mounted diode laser to produce 4 laser burns positioned at the posterior pole of the fundus positioned around and approximately 3 disc diameters from the optic disc. Laser settings included combinations of 50 μm and 100 μm diameter with 100 mW, 150 mW and 200 mW power. Pulse duration was 100 msec in all cases. In each eye, all 4 laser lesions were produced by similar combinations of laser settings. A total of 172 laser lesions were evaluated in 43 eyes. The immediate effects at the site of laser injury were noted. Specifically, the production of a chalk-white burn of the retina at the site of laser injury, the formation of a subretinal bubble of gas and any development of retinal or choroidal haemorrhage were documented. The incidence, extent and progression of any subsequent development of choroidal neovascularisation were evaluated by means of *in vivo* fluorescein angiography 2 weeks, 5 weeks and 8 weeks following laser injury.

The effects of different laser settings on immediate burn characteristics and the incidence of subsequent choroidal neovascularisation are summarised in Table 4.1. Laser injury at all combinations of laser settings invariably resulted in the immediate formation of chalk-white retinal blanching. The additional formation of a subretinal bubble of gas was observed most consistently using highest power and smaller spot size (Group C). The development of retinal haemorrhage at the laser site was observed only a small proportion of all groups at higher power settings. The use of high laser power and short pulse duration (Group C) also resulted in the highest incidence of choroidal neovascular lesions per burn. The immediate formation of a bubble of gas at the moment of laser injury was highly predictive for the subsequent formation of a neovascular lesion; the

presence of a bubble was associated invariably with the subsequent development of a lesion and conversely the absence of bubble formation was invariably associated with the lack of lesion formation.

Table 4.1 Effect of laser settings on lesion characteristics and incidence of subsequent choroidal neovascularisation

Group	No. of lesions	Laser power (mW)	Spot size (μ m)	Pulse duration (msec)	White-ning (%)	Bubble (%)	Haem (%)	CNV (%)
A	40	100	50	100	100	0.0	0.0	0.0
B	40	150	50	100	100	32	18.	32.5
C	24	200	50	100	100	100	25.	100
D	32	150	100	100	100	34	22.	35
E	36	200	100	100	100	42	14	47

Haem, intraretinal or subretinal haemorrhage immediately following laser injury; CNV, fluorescein leakage consistent with choroidal neovascularisation 2 weeks following laser injury

The effect of different laser settings on the extent of choroidal neovascularisation was determined by measuring the area of lesion-associated hyperfluorescence on *in-vivo* fundus fluorescein angiograms (Figure 4.7). Since adjacent neovascular lesions in the same eye occasionally became confluent the area of neovascularisation was analysed in terms of total area of hyperfluorescence per eye rather than areas of individual lesions. The extent of laser-induced choroidal neovascularisation area was clearly related to laser power. The use of higher laser power resulted in the greatest extent of lesions in terms of their mean area of hyperfluorescence (Group C) while laser at the lowest power settings induced no detectable lesions after 2 weeks (Group A). The range of values of neovascular area for each combination of settings was proportionally smaller for higher laser powers. Repeated fundus fluorescein angiography of the same animals 5 weeks and 8 weeks after laser injury demonstrated spontaneous regression of the induced choroidal neovascularisation. The reduction in area of neovascularisation between weeks 2 and 8 following laser injury was statistically significant in Groups C and E ($p < 0.01$).

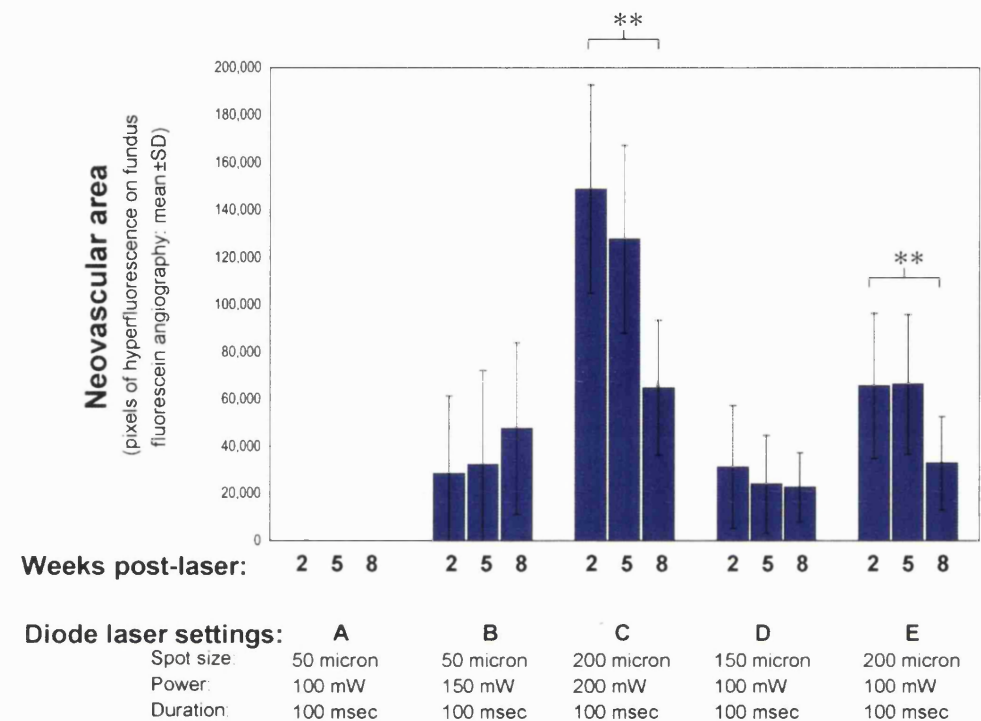


Figure 4.7 *Extent of experimental neovascular lesions induced by different combinations of laser settings over time*

The extent of neovascularisation is measured in terms of pixels of hyperfluorescence on digitised in vivo fluorescein angiograms. In order to characterise the evolution of induced neovascularisation over time, each combination of laser settings, angiograms were performed 2, 5 and 8 weeks following laser injury. (** $p < 0.01$)

The settings of Group C (200 μ m, 50mW, 100msec) resulted in the highest incidence of choroidal neovascular lesions per burn, the largest mean area of hyperfluorescence per eye and the proportionally smallest spread of values for lesion area. For these reasons this combination of settings was selected as the optimal combination for the most reproducible model for subsequent use in the evaluation of potential angiostatic therapies.

4.2 AAV-mediated and lentivirus mediated gene transfer in laser-induced choroidal neovascularisation

Having established and optimised a mouse model of laser-induced choroidal neovascularisation, this model was used as a context in which to evaluate reporter gene transfer following subretinal delivery of AAV and lentiviral vectors. AAV vectors are excellent candidates for gene transfer in the treatment of choroidal neovascularisation since they efficiently and stably transduce photoreceptors and retinal pigment epithelial cells, both of which are in close proximity to the developing neovascular complex. Lentiviral vectors are also attractive candidates because they have the ability to stably transduce non-dividing cells, and are deleted for all potentially pro-inflammatory viral components. Similarly, efficient transduction of RPE cells and modest transduction of photoreceptor cells has been reported after subretinal injection of HIV-based lentiviral vectors.^{260,261,348}

4.2.1 Production of AAV and lentiviral vectors

The production of recombinant AAV vectors and VSV-G pseudotyped lentiviral vectors is described in Chapter 2. Briefly, recombinant AAV (serotype 2) plasmids expressing green fluorescent protein (GFP) under the control of a cytomegalovirus (CMV) promoter (pHAV 5.5) were produced using a replicating amplicon system²²⁸ The titre of injected rAAV vector suspension, determined by infectious unit assay on 293 cells and by dot blot for DNA particles, was 5×10^9 infectious units/ml; 2×10^{11} particles/ml.

VSV-G pseudotyped lentiviral vectors were produced by transient transfection of three plasmids into 293T cells²⁵⁴ as described in Chapter 2. Briefly, the transfer vector used in this study was generated by modification of a basic self-inactivating vector pHR'SIN-CE (which itself incorporates a CMV promoter and enhanced green fluorescent protein (eGFP) reporter gene²⁵⁴) with the woodchuck post-transcriptional regulatory element (WPPE), and the central polypurine tract cis-acting sequence (cPPT) and central

termination sequence (CTS) of HIV-1. Lentiviral vector titres were approximately 2×10^8 transducing units per ml. Lentiviral vectors were produced and titrated by Ajay Mistry.

4.2.2 Subretinal vector delivery in mice

Delivery of vector suspensions into the subretinal space of mice was performed as described in Chapter 2. Briefly, mice were anaesthetised by intraperitoneal injection

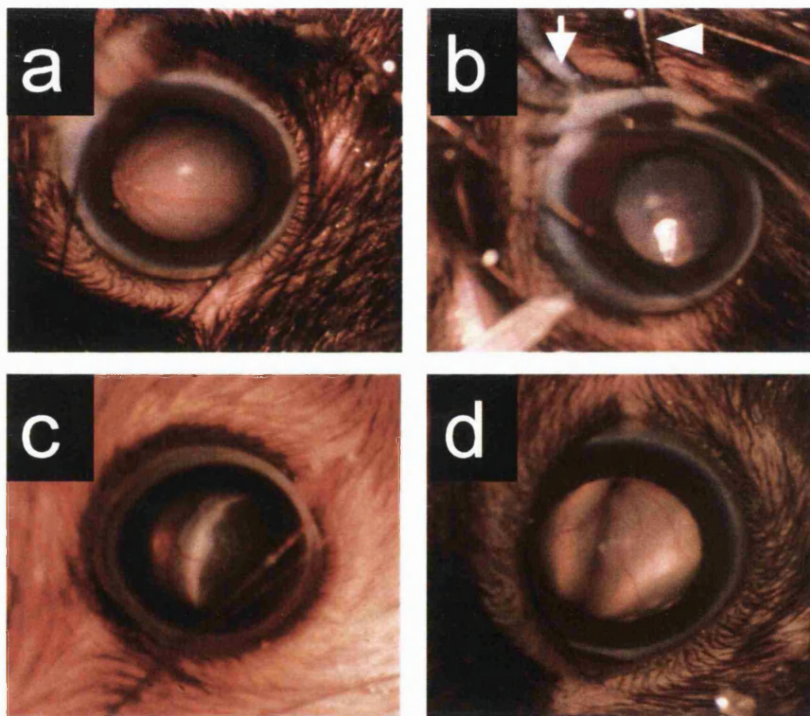


Figure 4.8 Delivery of vector suspension to the subretinal space in mice

a, View of globe through operating microscope. A plano contact lens system (not evident in this figure) comprising hydroxy-propyl-methyl cellulose and a glass coverslip facilitates a clear view of the retina; b, The globe is held in position using forceps (arrow) and the microsyringe needle is passed tangentially through the sclera into the subretinal space. The needle shaft is indicated (arrowhead) and the reflective tip can be seen in the subretinal space; c, injection of vector suspension causes a retinal 'bleb' to be raised; d, a second bleb is raised on the opposite side of the same eye in order to maximise the area of retinal transduction.

of 0.2ml Hypnorm and Hypnovel mixed 1:1:6 with PBS. Pupillary mydriasis was induced using topical 1% tropicamide. The injection procedure was performed under direct vision by means of an operating microscope using a 5 mm 34-gauge needle mounted on a 10 μ l Hamilton syringe (Figure 4.8). The fundus was visualised by means of a contact lens system consisting of a drop of 1% hypromellose solution on the cornea, covered with a glass coverslip. The needle tip was advanced through the sclera at the equator into the subretinal space, injection of viral suspension causing a localised retinal detachment as previously described.²³⁵ A volume of approximately 2 μ l of viral suspension was delivered into the subretinal space. The area of outer retinal reporter gene expression following subretinal vector delivery in mice extends only minimally beyond the original bleb of vector administration.^{243,261} In order to maximise the area of outer retinal transduction in this study, a further subretinal injection of vector suspension was performed in each eye, to create a second retinal bleb opposite the first. Vector titres were 2×10^{10} transducing units for AAV.CMV.GFP and 2×10^8 transducing units per ml for lenti.GFP.

4.2.3 Laser-induction of choroidal neovascularisation

To compensate for the expected delay in AAV mediated expression,²⁴³ laser-induction of choroidal neovascularisation was performed 6 weeks following subretinal AAV vector delivery. Since lentivirus-mediated transduction is established more rapidly²⁶⁰ laser-injury was performed only 2 weeks following subretinal lentiviral vector injection.

4.2.4 Evaluation of GFP reporter gene expression

Mice were sacrificed for analysis 2 weeks following laser injury. Cryosections of enucleated eyes were counterstained with propidium iodide and examined by fluorescence microscopy. Following subretinal delivery of AAV vector, efficient GFP reporter gene expression was evident in cells of the photoreceptor and retinal pigment epithelial layers. Sites of laser injury were evident as cellular masses in the subretinal

space stained by propidium iodide. Efficient GFP reporter gene expression was evident both in photoreceptor cells overlying the lesions and in retinal pigment epithelial cells adjacent to the lesions (Figure 4.9a)

Following subretinal delivery of lentiviral vector, efficient GFP reporter gene expression was evident in cells of the retinal pigment epithelium but not in photoreceptor cells. Transduced RPE cells were evident adjacent to the cellular proliferations at sites of laser injury (Figure 4.9b). These cells were typically seen lining the outer (choroidal) surface of the lesion but were also seen apparently enveloping its peripheral edge and extending over its inner, neurosensory aspect.

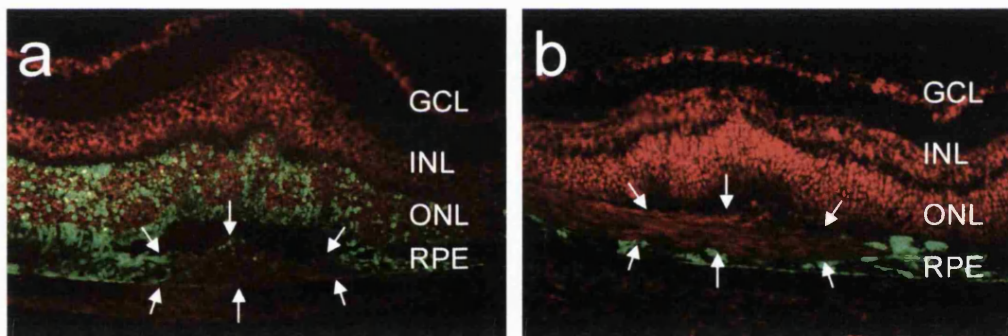


Figure 4.9 *GFP reporter gene expression in laser-induced choroidal neovascularisation two weeks following subretinal injection of vector*

a, Following subretinal injection of AAV.CMV.GFP, GFP fluorescence is evident in cells of the outer nuclear layer and retinal pigment epithelium in close proximity to the laser-induced lesion (arrows); b, Following subretinal injection of lenti.CMV.GFP, GFP fluorescence is evident across a large area of retinal pigment epithelium underlying and enveloping the laser-induced lesion (arrows) but not in the outer nuclear layer. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

4.3 AAV-mediated gene transfer of the soluble VEGF receptor sFlt-1 in experimental choroidal neovascularisation

Having demonstrated that AAV vectors offer an effective means to achieve efficient gene expression in close proximity to the developing laser-induced neovascular complex, further experiments were conducted to evaluate the effect of VEGF inhibition by AAV-mediated gene transfer of the soluble receptor sFlt-1 on the extent of experimental choroidal neovascularisation and to investigate the possibility of adverse effects in normal animals.

4.3.1 Evaluation of angiostatic effect of rAAV-mediated sFlt-1 expression in experimental choroidal neovascularisation

A recombinant AAV vector expressing the soluble truncated VEGF receptor sFlt-1 was produced and expression of sFlt-1 *in vitro* was confirmed by Western blot analysis as previously described. A similar vector expressing the reporter gene GFP was used as a control. The titres of vector preparations, as determined by dot-blot, were 2×10^{10} particles/ml in each case. These vectors were delivered to the subretinal space of mice aged 6 weeks as described above. Additional control eyes were injected using PBS or were uninjected. To compensate for the expected delay in AAV-mediated gene expression the vectors were delivered 6 weeks prior to induction of choroidal neovascularisation by laser rupture of Bruch's membrane. Using the slit-lamp mounted diode laser system, 4 laser burns were applied (100 mW, 100 μ m, 100 msec) to each retina at the posterior pole around the optic disc to create an immediate chalk-white blanching of the retina and the formation of a subretinal bubble of gas. The extent of subsequent choroidal neovascularisation 2 weeks later was determined by image quantification of the sum of areas of hyperfluorescent lesions per eye on *in vivo* fluorescein angiography. The mean areas of choroidal neovascularisation for each of the

treatment groups were compared and the statistical significance of their differences determined by Student's t-tests with Bonferroni's correction for multiple analyses. p -values ≤ 0.05 were considered statistically significant.

The mean areas (pixels $\times 1000 \pm \text{SEM}$) of hyperfluorescence per eye were 141.2 ± 18.8 in uninjected eyes ($n=25$), 142.9 ± 36.0 after subretinal injection of PBS, 185.1 ± 27.1 following injection of AAV.CMV.GFP and 97.1 ± 12.3 following injection of AAV.CMV.*sFlt-1* (Figure 4.10). Subretinal delivery of AAV.CMV.*sFlt-1* resulted in a reduction in the extent of choroidal neovascularisation by 31% compared to uninjected eyes ($p=0.06$) and by 48% compared to eyes injected with AAV.CMV.GFP ($p=0.01$). Other differences between groups were not statistically significant.

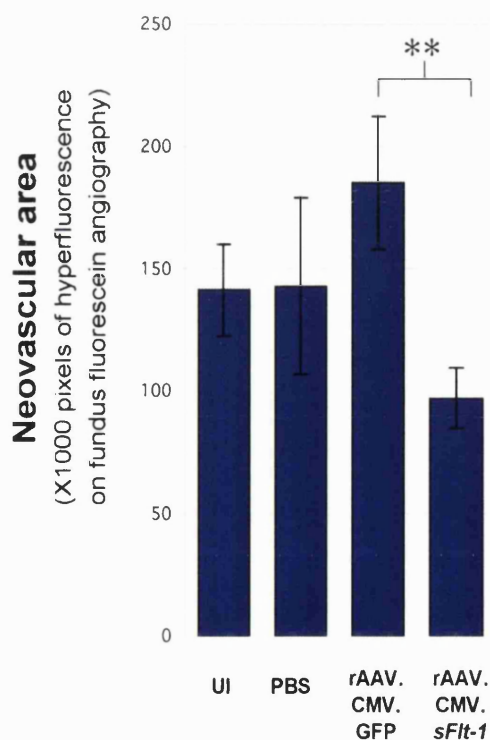


Figure 4.10 Effect of subretinal delivery of AAV.CMV.*sFlt-1* on laser-induced choroidal neovascularisation UI, uninjected; PBS, injected with phosphate-buffered saline. (** $p < 0.01$)

4.3.2 Long-term evaluation of potential adverse effects of AAV-mediated sFlt-1 expression in normal animals

In order to determine whether AAV-mediated local gene transfer of the VEGF inhibitor sFlt-1 results in adverse effects, further studies were performed to evaluate the long term effect of subretinal vector delivery on retinal vasculature and architecture in normal animals.

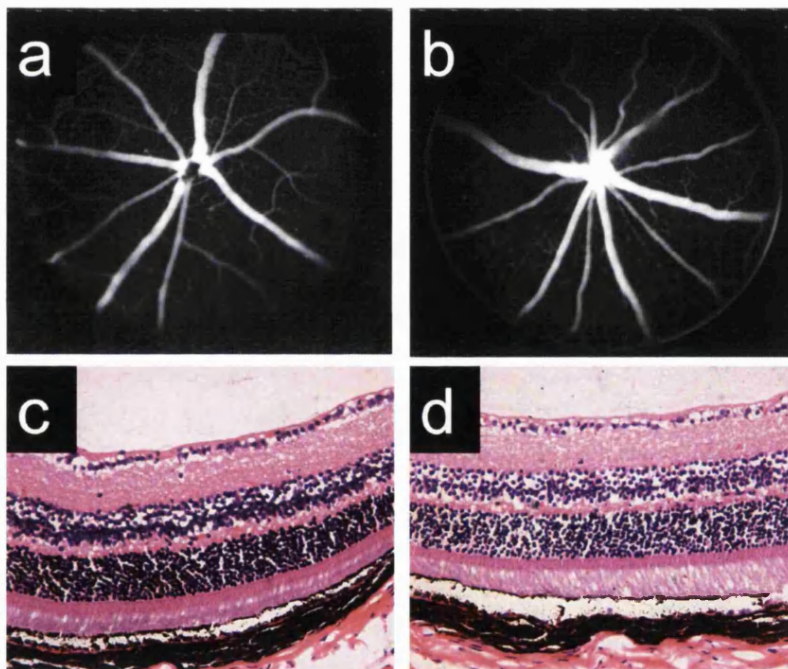


Figure 4.11 Effect of AAV.CMV.sFlt-1 in normal animals

Representative in-vivo fluorescein angiograms (a,b) and H&E-stained retinal sections (c,d) in normal uninjected animals (a,c) and 12 months following subretinal injection of AAV.CMV.sFlt-1 in normal animals (b,d). No adverse effects of long-term expression of sFlt-1 on retinal vasculature or architecture are apparent.

AAV.CMV.*sFlt-1* was injected subretinally in eyes of normal 6 week old C57Bl6/J mice (n=8) as previously described. Control eyes were uninjected (n=2). *In vivo* fluorescein angiography was performed when the animals reached 12 months of age. Animals were subsequently sacrificed for histological analysis. Light microscopy of 6 µm haematoxylin and eosin-stained sections was performed to evaluate any abnormalities of retinal architecture and to determine the presence of any inflammatory cell infiltrate or retinal thickening.

Twelve months following subretinal injection of AAV.CMV.*sFlt-1* in normal animals, *in vivo* fluorescein angiography demonstrated no significant structural or functional abnormality of retinal vasculature compared to uninjected control eyes. A discrete area of vascular irregularity was apparent in a minority of injected eyes, restricted to the site of the needle track. Light microscopy of retinal sections revealed normal retinal structure with no apparent change in retinal thickness and no evidence of an inflammatory cell infiltrate (Figure 4.11).

4.5 Discussion

4.5.1 Laser-induced murine model of choroidal neovascularisation

On the basis that laser-induced choroidal neovascularisation reflects the upregulation of VEGF expression and defects in Bruch's membrane that characterise the human disorder, a model of laser-induced choroidal neovascularisation in mice was established and refined in order to optimise its reproducibility with a view to the evaluation of angiostatic intervention.

Choroidal neovascularisation represents a stereotypic, nonspecific wound repair response independent of the underlying disease.¹⁶ In the present study both histological and

angiographic features of the experimental lesions were consistent with such a response to laser injury. Histological evaluation demonstrated a cellular proliferation in the subretinal space and immuno-staining for endothelial cells confirmed that this response included a significant vascular component. In-vivo fluorescein angiography demonstrated areas of hyperfluorescence at sites of previous laser injury that increased in brightness and extent following fluorescein injection, consistent with the clinical features of choroidal neovascularisation. Fluorescein-dextran perfused choroidal flatmounts clearly demonstrated abnormal complexes of vessels that were derived from the choroidal vasculature consistent with previous reports.^{338,349} Techniques for quantifying the extent of neovascularisation in fluorescein-dextran perfused choroidal flatmounts have been described³⁴⁴ but the preparation of these tissue specimens from mouse eyes is labour-intensive and, in contrast to in vivo fluorescein angiography, cannot be repeated in the same animal. The area of hyperfluorescence at the site of laser injury on in vivo fluorescein angiography was selected as the main outcome measure for this study since the leakage of fluorescein from immature vessels is a clinically relevant feature that reflects the angiogenic activity of the neovascular process. Although techniques to quantify the extent of neovascularisation by image analysis of vascular-specific staining in serial sections have also been described,³⁰² these methods are labour intensive and were not expected to offer an advantage for the purposes of the present study.

The laser settings determined the configuration of hyperfluorescence at sites of laser injury. Whereas lower power laser-injury resulted in the development of round disc-like lesions, the use of higher power laser produced lesions that typically comprised a ring of hyperfluorescence with a hypofluorescent centre. This observation is consistent with histological studies of laser-induced choroidal neovascularisation in primates that describe the presence of a central area of necrotic RPE/Bruch's /choroidal necrosis surrounded by a ring of damaged retinal pigment epithelium.^{350,351} An inflammatory response with infiltration of macrophages is followed by fibrovascular proliferation that appears to originate from the viable edges of this necrotic area and to extend through the break in Bruch's membrane into the subretinal space. The neovascular complex then undergoes envelopment by retinal pigment epithelial cells and spontaneous involution,

reflected by the cessation of visible fluorescein leakage on fundus fluorescein angiography.³⁵⁰

The use of a laser delivery system attached to a head-mounted indirect ophthalmoscope resulted in chalk-white blanching of the retina in the majority of burns but achieved less consistent immediate effects than the slitlamp-mounted alternative. This inconsistency is believed to be the result of the inability to standardise the spot size except through the subjective impression of the aiming beam diameter by the operator. Although the observation of a bubble of subretinal gas at the time of laser-injury has been interpreted as an indication of Bruch's membrane rupture,¹¹⁷ there is little evidence to support this. While no attempt was made in the present study to investigate the effect of laser injury on the integrity of Bruch's membrane, the formation of a bubble was found to be strongly predictive of the subsequent development of a neovascular lesion. Although bubble formation may not be direct evidence of Bruch's rupture, it does appear to correlate closely with the degree or nature of injury required to induce choroidal neovascularisation. Since burns unassociated with immediate bubble formation are unlikely to result in detectable neovascular lesions, their exclusion from the subsequent analysis presents one means to improve the reliability of the model.

The use of image processing software enabled quantification of the area of hyperfluorescence at sites of laser injury. Evaluation of the effects of a number of combinations of laser settings identified a combination that resulted predictably in a high incidence of neovascular lesions of large area and relatively narrow spread. This combination of settings was used in subsequent experiments to investigate the angiostatic effect of candidate molecules.

4.5.2 AAV-mediated and lentivirus-mediated reporter gene transfer in laser-induced choroidal neovascularisation

This study demonstrated that both AAV and lentiviral vectors mediate efficient transduction of cells in close proximity to the fibrovascular lesion induced by laser

rupture of Bruch's membrane in this model. While subretinal delivery of the AAV vector resulted in transduction of both photoreceptors and retinal pigment epithelial cells, delivery of the lentiviral vector specifically transduced retinal pigment epithelial cells. These results are consistent with previous reports that describe lentiviral transduction of photoreceptors that is inefficient and occurs only in newborn animals or at the immediate area of needle trauma at the injection site.^{260,261,348} Following laser-induction of choroidal neovascularisation, lentivirus-transduced retinal pigment epithelial cells were typically evident lining the outer (choroidal) surface of the lesion but were also seen apparently enveloping its peripheral edge and extending over its inner, neurosensory aspect. The proliferation of retinal pigment epithelial cells in this context and their envelopment of newly formed vessels is well described³⁵⁰ and is believed to represent an attempt to resist progressive neovascularisation and to induce spontaneous involution. Vector-mediated expression of an angiostatic factor by these cells is an attractive scenario that might optimise their ability to arrest a neovascular response.

Both AAV and lentiviral vectors may be valuable for the therapeutic gene transfer of anti-angiogenic factors in the context of choroidal neovascularisation. While AAV vectors may result in a greater number of transduced cells in close proximity to a subretinal neovascular lesion, the relative specificity of lentiviral vectors for retinal pigment epithelial cells may offer adequate expression without the potential of adverse effects due to transduction of photoreceptor cells. Although the use of HIV-based lentiviral vectors raises concerns about recombination events leading to the generation of dangerous replication-competent mutants, extensive sequence manipulation and deletion within the vector genome virtually eliminates this possibility.²⁵⁴ The use of lentiviral vectors such as feline immunodeficiency virus and equine infectious anaemia virus, which are non-pathogenic to humans may offer an even safer alternative.

Alternative vectors that have been utilised for gene-based therapy of choroidal neovascularisation include adenoviral and retroviral systems. Adenoviral vectors mediate highly efficient intraocular expression following subretinal or intravitreal vector delivery and have effectively delivered angiostatic molecules in experimental models.^{75,352} The

immunogenicity and short-lived expression associated with most adenoviral vectors, however, mean that these are poorly suited to applications in chronic or recurrent disease processes such as choroidal neovascularisation. Retroviral vectors have been utilised for their ability to target dividing cells. These vectors can specifically target proliferating endothelial cells at sites of laser injury in models of choroidal neovascularisation,³⁵³⁻³⁵⁵ but this strategy is not likely to protect against subsequent neovascular episodes without repeated vector administration. In contrast, studies of AAV and lentivirus systems suggest that both these vectors are likely to mediate expression well beyond the period of active neovascularisation in experimental models of short-lived choroidal neovascularisation and to mediate sustained expression that might offer protection in the long-term.

4.5.3 AAV-mediated gene transfer of the soluble VEGF receptor sFlt-1 in experimental choroidal neovascularisation

In these experiments described above, subretinal injection of AAV.CMV.*sFlt-1* resulted in a 31 % reduction in the extent of laser-induced choroidal neovascularisation compared to untreated eyes. In a similar study the effect of subretinal delivery of AAV.CMV.*sFlt-1* on the incidence and neovascular activity of laser-induced neovascular lesions was evaluated in a rat model. Although the models and outcome measures are slightly different to those used in the present study, the results are broadly comparable with a 19% reduction in the number of laser-induced lesions that formed new vessels and a 22% reduction in fluorescein leakage from lesions compared to control eyes in which AAV.CMV.GFP was injected.³⁵⁶ As is the case in experimental retinal neovascularisation described in Chapter 3, residual neovascularisation may be the result of either incomplete inhibition of VEGF or of an uninhibited independent parallel pathway. The finding that choroidal neovascularisation can be dramatically inhibited by oral administration of a VEGF specific protein kinase inhibitor suggests that VEGF signaling plays a critical role in the development of CNV in this model^{342,357} and implies that the efficacy of a VEGF-directed intervention may be improved by optimising the delivery system.

Alternative molecular pathways involved in the pathogenesis of ocular angiogenesis include PEDF,⁹⁷ MMPs,¹³² angiostatin,¹⁵³ endostatin¹⁶² and the angiopoietins¹⁶⁹ though many of these mechanisms are not independent of the VEGF pathway. PEDF is believed to be the major natural angiogenesis inhibitor in the vitreous⁹⁷ and is an attractive alternative therapeutic candidate. A critical balance between PEDF and VEGF has been described such that PEDF counteracts the angiogenic potential of VEGF; down-regulation of PEDF expression leads to an equilibrium shift that promotes neovascularisation.³⁵⁸ Although the author's own attempts to inhibit choroidal neovascularisation in this model by rAAV-mediated CMV-driven expression of PEDF have not yet proven successful, other groups have demonstrated that this factor has valuable therapeutic potential. Subretinal injection of an adenoviral vector expressing PEDF not only reduces progression of experimental choroidal neovascularisation⁹⁶ but also accelerates its spontaneous regression by promoting apoptosis³⁵⁹ PEDF expression mediated by a AAV vector under the control of a chicken beta actin (CBA) promoter also results in significant reduction the extent of experimental choroidal neovascularisation.³⁶⁰ Since both adenovirus-mediated and CBA-driven AAV-mediated expression are highly efficient, one possible reason for our lack of success using AAV.CMV.PEDF may be sub-optimal expression. Studies are ongoing to construct a AAV vector to deliver PEDF under the control of a CBA promoter and to identify a reliable antibody to confirm and quantify PEDF expression.

The results of the present studies demonstrate efficient lentivirus-mediated reporter gene expression in retinal pigment epithelial cells at the site of experimental neovascular lesions. Although the author has found that lentivirus-mediated expression of sFlt-1 inhibits angiogenesis in a tube-forming assay *in vitro*, attempts to inhibit experimental choroidal neovascularisation by lentivirus-mediated expression of sFlt-1 have not yet proven successful. One possible reason for the current lack of success may be sub-optimal titres of vector suspensions and work is ongoing to address this by the production of high-titre lentiviral stocks.

Effective local delivery of soluble angiostatic factors may be achieved by transduction of cells in close proximity to the neovascular lesion or alternatively by diffusion from transduced cell populations at a more distant location. In the present study vector delivery was performed over a wide area across the posterior pole of each eye such in order to transduce retinal cells in immediate proximity to the induced neovascular lesions. In a similar study performed by another group, subretinal delivery of a AAV.CMV.*sFlt-1* (of comparable titre) to a site in the retinal periphery distant to the site of laser-rupture resulted in a more modest angiostatic effect.³⁵⁶ While vector delivery to the retinal periphery in this way avoids the need for surgically-induced macular detachment, the distance required for diffusion of soluble factor may result in an attenuation of the potential angiostatic efficacy. Submacular surgical techniques for choroidal neovascularisation offer an opportunity to transduce cells at the site of neovascular lesions without creating a macular detachment solely for the purpose of vector delivery.

4.5.4 Evaluation of toxicity due to rAAV-mediated gene transfer of sFlt-1 in normal animals

Subretinal delivery of AAV.CMV.*sFlt-1* in the normal mature murine retina resulted in no apparent adverse effect on retinal architecture or vasculature. Although VEGF has an important role as a survival factor for immature retinal capillary endothelium³¹⁹ the results of this study suggest that this may not be the case in adult retina. The absence of an inflammatory cell infiltrate is only an indication that there is no active ongoing local immune response to the vector or the expressed protein but this possibility could be investigated further by immuno-phenotyping at a number of different time points, for example to identify markers of microglial or T cell activation. In addition to acting as a survival factor for immature retinal capillary endothelium VEGF may also have a neuroprotective role.³⁶¹ The possibility that long-term VEGF inhibition may result in loss of retinal neurones cannot be excluded by conventional histology but work is ongoing to evaluate the effects of long-term expression of sFlt-1 on neuro-retinal function by electrophysiological evaluation.

Although these results demonstrate that subretinal injection of AAV.CMV.*sFlt-1* in normal animals resulted in no apparent adverse effects, significant expression of sFlt-1 protein by ELISA of pooled eye homogenates 8 weeks following vector delivery has not yet been demonstrated. Until sustained expression of the protein *in vivo* can be demonstrated unequivocally these results must be interpreted with caution. The demonstration of sFlt-1 expression by Western analysis following transfection by AAV.CMV.*sFlt-1* plasmid of 293 cells *in vitro* (Chapter 3, section 3.4.1.3) confirms that the construct is being transcribed, and the production of high-titre vector preparations is evidence that it is effectively packaged into viral particles. The significant reduction in extent of choroidal neovascularisation following subretinal delivery of AAV.CMV.*sFlt-1* described in this chapter is also consistent with an angiostatic effect due to local sFlt-1 expression. An angiostatic effect following subretinal delivery of AAV.CMV.*sFlt-1* in a rat model of choroidal neovascularisation has also been described.³⁵⁶ Though Western blot analysis of culture supernatant from virus-infected cells confirmed the expression of sFlt-1 *in vitro*, and RT-PCR of chorioretinal tissue suggested transcription of sFlt-1 *in vivo*, quantification of protein levels was not reported in this study.

There are a number of reasons that might explain why ELISA of pooled eye homogenates following subretinal delivery of AAV.CMV.*sFlt-1* has failed to convincingly demonstrate sFlt-1 expression and further studies are ongoing to investigate these possibilities. One possibility is that the quantity of sFlt-1 expressed by AAV in small numbers of mouse eyes is too low for the sensitivity of the ELISA used. A more sensitive technique such as quantitative PCR might detect transcription more reliably. Another possible reason for the failure to detect sFlt-1 *in vivo* is that an immune response directed against human sFlt-1 might reduce levels of available protein. Although a sandwich ELISA for antibodies to human sFlt-1 has not demonstrated a significant humoral immune response, this possibility is being investigated further by vector delivery in immuno-compromised nude mice. A third possibility is that the AAV vector preparations that were used in toxicity experiments were produced in the latter stages of this project may have an unexpectedly low infectious titre despite a high particle titre, possibly resulting from changes in the method of vector preparation. This possibility is currently

being addressed by a detailed review of vector production techniques. Once sustained AAV-mediated expression of sFlt-1 has been reliably demonstrated in the long-term these toxicity studies will be repeated in both rodents and higher animals.

The results of studies described in this chapter support the principle that local AAV-mediated gene transfer of sFlt-1 offers a potentially valuable novel approach to the clinical management of choroidal neovascular disorders. Whilst the results to date have identified no evidence of significant toxicity the development of strategies to control gene expression would offer an attractive refinement and such a strategy is described in Chapter 5. Conclusive demonstration of sFlt-1 expression *in vivo* is required before further pre-clinical toxicity studies and clinical trials can be initiated. The extension of this approach to clinical trials also requires a thorough evaluation of AAV-mediated retinal gene transfer in intermediate models and this is the subject of Chapter 6.

Acknowledgements

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Regulation of gene expression in experimental retinal and choroidal neovascularisation

The chapter describes the evaluation of a novel strategy to enable the appropriate regulation of transgene expression in experimental retinal and choroidal neovascularisation.

5.1 Introduction

A major advantage of a gene transfer approach to the delivery of angiostatic proteins in ocular neovascular disorders is the possibility of sustained local expression following a single procedure to deliver a vector into the eye. Previous chapters describe how inhibition of vascular endothelial growth factor (VEGF) by AAV-mediated local gene transfer of its soluble receptor sFlt-1 can lead to a reduction in the extent of neovascularisation in murine models of both ischaemic retinopathy and neovascular age-related macular degeneration.³⁶² Similar therapeutic effects have been described in experimental models by local gene transfer of alternative angiostatic factors including pigment epithelium derived factor⁹⁶, TIMP-3,³⁴⁵ endostatin³⁵² and angiostatin.³⁰² Many molecular mediators of angiogenesis, however, mediate essential physiological functions in the retina. In addition to its role in physiological and pathological angiogenesis, VEGF is acts as a survival factor for vascular endothelium,³¹⁹ maintains the integrity of the fenestrated epithelium of the choriocapillaris³⁶³ and may have a further supportive role for

for neuronal cells.³⁶¹ For this reason the long-term uncontrolled inhibition of VEGF by the sustained and unregulated expression of an inhibitor presents a significant risk of adverse local effects. The development of strategies to achieve effective targeting of transduction and to enable appropriate regulation of gene expression are therefore required to minimise the potential for local toxicity.

The incorporation of a hypoxia response element (HRE) into promoter sequences of therapeutic constructs results in the regulation of transgene expression in response to hypoxia. An HRE is a highly conserved *cis*-acting specific enhancer, present in a number of genes including those encoding VEGF, erythropoietin and inducible nitric oxide synthase, that mediates transcriptional responses to hypoxia following binding by the transcription factor Hypoxia-inducible factor-1 (HIF-1).³⁶⁴ HIF-1 is composed of two basic helix-loop-helix PAS-proteins: HIF-1 beta/ARNT (aryl hydrocarbon receptor nuclear translocator), which is constitutively expressed, and HIF-1 alpha, which is degraded by the ubiquitin-proteasome system in normoxia but stabilised under hypoxic conditions. HRE-regulated approaches are currently being pursued in the design of vectors for hypoxia-targeted delivery in several contexts. Hypoxia-inducible vectors are of potential value for tumor-targeting in cancer gene therapy.³⁶⁵ Similar systems have been described to automatically up-regulate expression of cardioprotective transgenes in myocardial ischaemia³⁶⁶⁻³⁶⁸ and to achieve physiological regulation of erythropoietin expression to appropriately correct anaemia in a rodent model of erythropoietin deficiency.³⁶⁹

This chapter describes a strategy to target and appropriately regulate AAV-mediated gene expression in experimental retinal and choroidal neovascularisation through the incorporation of an HRE promoter sequence.

5.2 HRE-driven gene expression in experimental retinal neovascularisation

HRE-driven reporter gene expression was first evaluated following intravitreal vector delivery in the murine model of ischaemia-induced VEGF-dependent retinal neovascularisation.

5.2.1 Construction of AAV.HRE.*GFP*

A synthetic hypoxia-responsive element, a kind gift from Oxford Biomedica, combines low basal expression in normoxic conditions with high-level activated expression when the oxygen concentration is low.³⁷⁰ A recombinant AAV (serotype 2) vector encoding the *GFP* gene under the control of the HRE promoter was constructed. The GFP cDNA was removed from pd2EGFP-1 (BD Biosciences Clontech UK, Oxford) as a *Sma*I-*Xba*I fragment. This was blunt cloned in to the *Hind*III-*Xba*I sites of OBHRE pGL3 plasmid³⁷⁰ to replace the luciferase gene and create pOBHRE.*GFP*. The *Bln*I-*Hpa*I GFP containing fragment from pOBHRE-GFP was cloned in place of the mEPO gene in AAV.HRE.*Epo*³⁶⁹ to create the AAV.HRE.*GFP* plasmid.

Recombinant AAV vectors (serotype 2) were produced using the replicating amplicon system previously described²²⁸ and purified by a one step affinity purification procedure on a heparin column.²²⁷ The titre of AAV particles were determined by dot blot quantification of genome copy and direct comparison to a recombinant AAV vector expressing CMV.*GFP* of known biological titre. Vector suspensions of comparable titre (1×10^{11} particles/ml) were used for all intra-ocular injections.

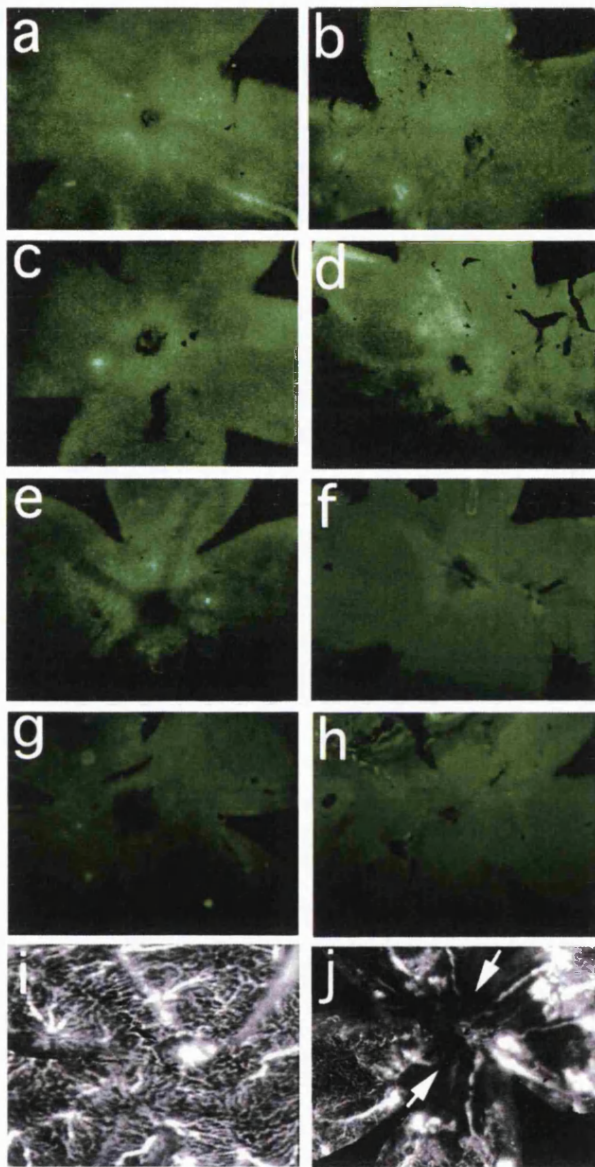
5.2.2 Delivery of AAV vectors in experimental retinal neovascularisation

Retinal ischaemia was induced in mice as described previously. Briefly, mouse pups were exposed to $75\pm 3\%$ oxygen from p7 to p12 in a sealed incubator along with nursing mothers to induce retinal capillary obliteration at the posterior pole. Age-matched control animals were raised in room air throughout.

In order to determine the pattern of HRE-driven transgene expression following intra-ocular administration of AAV vectors in this model, GFP reporter gene expression was evaluated following intravitreal injection of vectors in which transgene expression was driven by either HRE or CMV promoters. Eyes of mice raised in hyperoxia and control animals raised in room air were intravitreally injected using AAV.HRE.*GFP* (n=15), AAV.CMV.*GFP* (n=15) or were uninjected (n=8). To compensate for the delay in AAV-mediated expression and achieve established expression by the time of onset of active angiogenesis in this model (postnatal day p12 to p19), AAV vectors were delivered at p2. The injection procedure was performed as previously described. A volume of approximately 1 μ l of viral suspension was injected into the vitreous cavity in each case.

5.2.3 Analysis of GFP expression in retinal whole-mounts and cryosections

Animals were enucleated for analysis of reporter gene expression at p19 during the peak of ischaemia-induced retinal neovascularisation, or at p34 by which point a more normal vascular architecture is restored.³⁰⁸ Analysis of GFP reporter gene expression was performed in whole-mounted retinas and in retinal cryosections. Retinal whole-mounts and cryosections were prepared after fixation of enucleated eyes in 4% paraformaldehyde, as described in Chapter 2, and examined by fluorescence microscopy.



(a) CMV-driven expression in model of retinal ischaemia at post-natal day 19 (p19). (b) CMV-driven expression in model of retinal ischaemia at p34. (c) CMV-driven expression in normal control at p19. (d) CMV-driven expression in normal control at p34. (e) HRE-driven expression in model of retinal ischaemia at p19. (f) HRE-driven expression in model of retinal ischaemia at p34. (g) HRE-driven expression in normal control at p19. (h) HRE-driven expression in normal control at p34. In order to illustrate the typical pattern of retinal vascular non-perfusion in this model, fluorescent micrographs of retinal flatmounts from uninjected normal (i) and ischaemic (j) retinas following terminal perfusion with fluorescein-conjugated dextran at p19 are included. Retinal non-perfusion is evident as hypofluorescent areas devoid of normal retinal capillary network (white arrows).

Figure 5.1 CMV-driven and HRE-driven AAV-mediated GFP expression after intravitreal vector delivery in a murine model of retinal neovascularisation: retinal flatmounts
GFP expression is evident as bright granular green fluorescence on a background of diffuse green autofluorescence of photoreceptor cells.

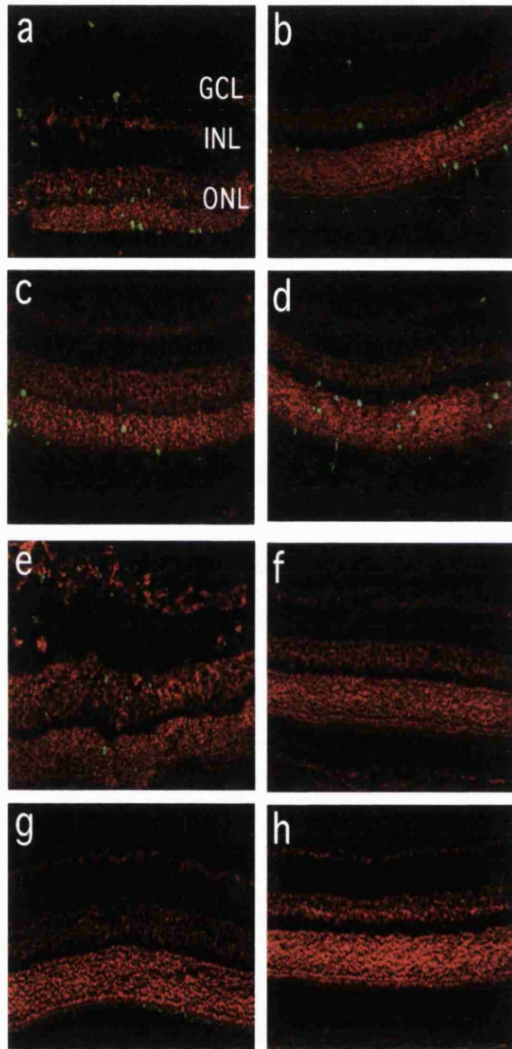


Figure 5.2 CMV-driven and HRE-driven GFP expression following intravitreal vector delivery in murine model of ischaemia-induced retinal neovascularisation: fluorescence micrographs of retinal cryosections
 (a) CMV-driven expression in model of retinal ischaemia at post-natal day 19 (p19). The ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL) are labelled. (b) CMV-driven expression in model of retinal ischaemia at p34. (c) CMV-driven expression in normal control at p19. (d) CMV-driven expression in normal control at p34. (e) HRE-driven expression in model of retinal ischaemia at p19. (f) HRE-driven expression in model of retinal ischaemia at p34. (g) HRE-driven expression in normal control at p19. (h) HRE-driven expression in normal control at p34.

After induction of retinal ischaemia, CMV-driven reporter gene expression at p19 was evident in cells widely distributed across the retina (Figure 5.1a), in the ganglion cell layer, inner nuclear and the outer nuclear layers (Figure 5.2a). At p34 CMV-driven expression was sustained across the retina (Figure 5.1b, 5.2b). In age-matched control mice without retinal ischaemia CMV-driven expression was similarly widespread (Figure

5.1c, 5.2c) and sustained (Figure 5.1d, 5.2d). In contrast, in mice with retinal ischaemia HRE-driven reporter gene expression at p19 was restricted to areas at the posterior pole (Figure 5.1e) in a configuration consistent with the typical pattern of retinal capillary non-perfusion in this model (Figure 5.1j). GFP expression was observed in the ganglion cell layer, inner nuclear and the outer nuclear layers (Figure 5.2e). At the later time-point (p34) in animals previously exposed to hyperoxia, HRE-driven GFP expression was observed only in occasional cells (Figure 5.1f, 5.2f). In age-matched control animals without retinal ischaemia HRE-driven expression was not observed at either time point (Figure 5.1g, 5.1h, 5.2g, 5.2h).

5.3 HRE-driven expression in experimental choroidal neovascularisation

HRE-driven reporter gene expression was next evaluated following subretinal vector delivery of AAV vectors in a murine model of laser-induced choroidal neovascularisation.

5.3.1 Delivery of AAV vectors in experimental choroidal neovascularisation

Choroidal neovascularisation was induced in adult mice by laser-rupture of Bruch's membrane as previously described. Briefly, diode laser retinal photocoagulation was performed in adult C57Bl/6J mice (200 mW, 100 μ m spot size, 100 msec) using the slit-lamp mounted delivery system previously described at 4 sites around the optic disc at the posterior pole to induce formation of an air bubble and an immediate chalk-white blanching of the retina. Age-matched control animals received no laser injury.

In order to determine the pattern of HRE-driven transgene expression in this model, GFP reporter gene expression was compared after subretinal delivery of vectors driven by HRE and CMV promoters. The AAV.HRE.GFP plasmid was constructed and vector

produced as previously described. Eyes of mice in which choroidal neovascularisation was to be induced by laser injury and unlasered control eyes were injected subretinally using AAV.HRE.*GFP* (n=12), AAV.CMV.*GFP* (n=12) or were uninjected (n=6). To compensate for the delay in AAV-mediated expression, vector delivery was performed 4 weeks prior to laser injury. Delivery of vectors to the subretinal space was performed as described in Chapter 2. For each eye the procedure was repeated at a second site opposite the original injection site in order to maximise the area of retinal transduction. A total of approximately 4 μ l of the vector suspension was injected into the subretinal space in each eye.

5.3.2 Analysis of GFP expression in retinal cryosections

Laser-induced rupture of Bruch's membrane results in the growth of new vessels that extend into the subretinal space from the choroid.³⁴¹ The neovascular response following laser-rupture evolves during the first two weeks after and thereafter is non-progressive or gradually regresses.³³⁵ In order to compensate for the expected delay in transgene expression and achieve established expression during the period of active angiogenesis in this model (the first two weeks after laser induction) the vector was delivered 4 weeks in advance of laser rupture. The eyes were analysed for vector-mediated expression immediately prior to laser rupture, 7 days after laser rupture during the period of active neovascularisation, and after 28 days at which point the choroidal neovascularisation is believed to be no longer active. Mice were sacrificed by cervical dislocation for analysis of GFP reporter gene expression in retinal cryosections as described above (Figure 5.3).

Laser injury resulted in the presence of a limited amount of autofluorescent debris in the subretinal space regardless of vector delivery (5.3b, 5.3c). After subretinal delivery of AAV, CMV-driven expression was observed in photoreceptor cells prior to laser injury (Figure 5.3d), 7 days after laser-injury (Figure 5.3e) and was sustained at 28 days (Figure 5.3f). GFP expression was widely distributed across the retina at sites of laser-rupture

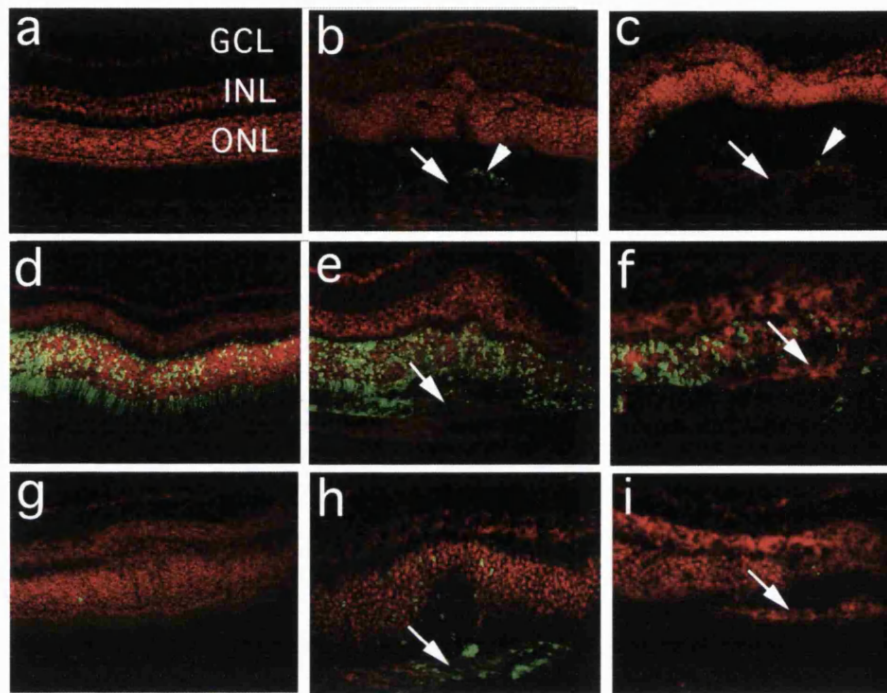


Figure 5.3 *CMV-driven and HRE-driven GFP in laser-induced choroidal neovascularisation: fluorescence microscopy of retinal cryosections*

(a) Retinal cryosections from uninjected eyes without laser-rupture of Bruch's membrane. The ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL) are labelled. (b) Sections 7 days after laser-rupture and (c) 28 days after laser rupture. Laser rupture resulted in a limited amount of autofluorescent material (white arrowheads) in the subretinal space regardless of vector delivery. (d) Sections from eyes following subretinal injection of AAV.CMV.GFP without laser-rupture, (e) 7 days following laser-rupture and (f) 28 days following laser-rupture. Laser-induced choroidal neovascular complexes are indicated (white arrows) (g) Sections from eyes following subretinal injection of AAV.HRE.GFP without laser rupture, (h) 7 days following laser-rupture and (i) 28 days following laser rupture.

(Figure 5.3e) and elsewhere. In contrast, significant HRE-driven GFP expression was evident only at sites of laser rupture and was not sustained over time. Prior to laser injury HRE-driven expression was evident in only occasional photoreceptors (Figure 5.3g). 7 days after laser injury HRE-driven expression was seen in significant numbers of cells overlying the site of laser injury (Figure 5.3h) but not elsewhere. After 28 days HRE-driven expression was observed only in occasional photoreceptors even at the site of laser rupture (Figure 5.3i).

5.4 Discussion

5.4.1 Targeting of cellular transduction and regulation of gene expression

The results of experiments in previous chapters demonstrate that AAV-mediated gene transfer of the VEGF inhibitor sFlt-1 reduces neovascularisation in short-lived animal models and offers a powerful approach to the local, sustained delivery of angiostatic proteins for the treatment of ocular neovascular disorders. The possibility that uncontrolled sustained expression of sFlt-1 may cause adverse effects in the long term might be minimised by the development of strategies for the targeting of cellular transduction and for the appropriate regulation of gene expression. Specific tissues may be targeted by an appropriate choice of vector and by the accurate delivery of vector suspension. Retinal pigment epithelial cells, for example, may be specifically targeted by lentiviral vectors delivered to the subretinal space.²⁶¹ Proliferating cells in any tissue may be targeted by retroviral vectors since these specifically transduce cells during cell division.^{245,248} The different time-courses of expression mediated by different vectors may be exploited to optimise the timing of therapeutic protein delivery. Adenovirus-mediated expression, for example, is rapid in onset but typically of limited duration as a result of the induction of an immune response.²¹³ AAV-mediated expression, on the other hand, is delayed in onset²⁷⁰ and subsequently sustained for months or years. Although vectors that

mediate only short-term expression, such as adenoviral vectors, may be less likely to cause long-term adverse effects, their limited duration of expression also risks an increased likelihood of disease recurrence.

The efficiency of gene expression by a transduced cell is modulated by promoter sequences and regulatory elements. The design and incorporation of these elements in therapeutic constructs offers a valuable opportunity to achieve appropriate control of transgene expression. Cell-specific promoters can be incorporated to restrict expression to a given cell type. The use of an opsin promoter, for example, results in efficient expression only in photoreceptor cells while minimising inappropriate expression from transduced retinal pigment epithelial cells. The timing and level of gene expression can be regulated through the incorporation of inducible promoters that are responsive to the administration of exogenous pharmacological agents. In such systems transgene expression can be induced, for example, by the systemic administration of a drug and the level of gene expression regulated by adjusting its dose according to therapeutic or adverse effects. Tetracycline-inducible systems, for example, can facilitate regulation of gene expression in the retina using adenoviral³⁷¹ or AAV³⁷² vectors. Intraocular expression following AAV-mediated transduction may similarly be regulated by rapamycin-inducible systems.³⁷³ These pharmacological approaches are dependent, however, on regular clinical observation to determine appropriate dosing and may be limited by inefficient drug penetration across the blood-retinal barrier.

An alternative strategy to regulate gene expression is to couple transgene expression to changes in the local tissue environment. Since retinal neovascularisation is typically the result of local tissue hypoxia, the possibility of hypoxia-regulated expression of angiostatic molecules by the incorporation of a hypoxia response element (HRE) presents an attractive scenario. A synthetic HRE developed by Oxford Biomedica combines low basal expression in normoxic conditions with high-level activated expression when the oxygen concentration is low.³⁷⁰ In this study the HRE was used to target AAV-mediated reporter gene expression to sites of experimental retinal and choroidal neovascularisation.

5.4.2 HRE-driven expression in experimental retinal and choroidal neovascularisation

In ischaemia-induced retinal neovascularisation CMV-driven expression was observed across a wide area of the retina and was sustained over time beyond the period of active angiogenesis. In contrast, HRE-driven expression was evident only at the posterior pole in a pattern that is consistent with the temporal and spatial features of retinal capillary non-perfusion.³⁰⁸ Furthermore, HRE-driven expression was not sustained beyond the period of hypoxia-driven, VEGF-induced angiogenesis that is well characterised in this model.⁵³ Thus AAV-mediated HRE-driven reporter gene expression is targeted both spatially and temporally to the areas of retinal ischaemia in this model.

Following laser rupture of Bruch's membrane, CMV-driven GFP expression was evident constitutively in photoreceptor cells and retinal pigment epithelial cells regardless of the timing or site of laser rupture. HRE-driven expression however was restricted to photoreceptor cells overlying the developing choroidal neovascular complex; expression was not significant prior to laser rupture and was not sustained after 4 weeks even at the site of laser rupture. Though laser rupture resulted in a limited amount of autofluorescent material in the subretinal space regardless of vector delivery, HRE-driven GFP expression was also observed in RPE cells specifically at sites of laser injury. While HRE-driven expression in this model is consistent with a possible role for hypoxia in the evolution of choroidal neovascularisation following laser rupture of Bruch's membrane, alternative mechanisms may be involved. HREs may be activated by transcription factors other than HIF-1³⁷⁴ and it is possible that HRE-driven expression may occur as part of a stress or wound-healing response in the context of laser injury. VEGF expression is well-described in laser-induced choroidal neovascularisation,^{333,334,357} and peaks at up to 7 days after wounding.³³⁵ Retinal VEGF expression may also be upregulated, however, in conditions other than ischaemia³⁷⁵ in an mechanism that is independent of hypoxia-induced activation of HIF-1. Further experiments to investigate the possible role of

hypoxia in this model using the bio-reductive marker nitro imidazole theophylline (NITP) are planned. Whatever is the mechanism in this experimental model, a role for ischaemia has been suggested in the pathogenesis of choroidal neovascularisation in human patients.^{17,18} Retinal hypoxia may result from thickening of Bruch's membrane or abnormalities of choroidal perfusion,¹⁴⁻¹⁶ leading to an upregulation of VEGF expression in retinal pigment epithelial cells that has been demonstrated *in vitro*³⁷⁶ and there is reason to believe that HRE-driven expression might facilitate the effective targeting of angiogenesis in the clinical context.

5.4.3 Conclusions

The results of these experiments demonstrate that HRE-driven AAV-mediated transgene expression *in vivo* specifically targets experimental retinal and choroidal neovascularisation both spatially and temporally and offers an attractive strategy for the targeted and regulated delivery of angiostatic proteins to the retina in the management of neovascular disorders.

In both models of neovascularisation described in this study, the neovascular response occurs in response to a single event and is relatively short-lived in duration. While the self-limiting course is dissimilar to retinal and choroidal neovascularisation in human disorders (which typically have a more prolonged course) in the present study the transient course enabled the investigation of HRE-driven expression in response to changes in the local tissue environment over time. In both models the incorporation of a HRE into the promoter sequence targets gene expression to sites of neovascularisation and results in expression that is not sustained beyond the period of active angiogenesis. This hypoxia-inducible system offers a powerful means to achieve spatial and temporal regulation of expression of angiostatic proteins in the management of retinal and choroidal neovascularisation by gene transfer. In this way sites of active neovascularisation may be effectively targeted, while minimising inappropriate expression elsewhere or during periods of angiogenic inactivity. This approach offers a

‘vigilant’ system in which HRE-driven expression is likely to anticipate the onset of a neovascular response to ischaemia in a way not possible with pharmacologically-inducible systems in which dosing is dependent on the clinical observation of established new vessels. This strategy also offers the possibility of disease-related dosing in which the level of HRE-driven expression is continuously adjusted in response to changes in tissue oxygenation such that it is directly correlated to the intensity of angiogenic drive.

Although in the absence of retinal ischaemia or laser injury the background HRE-driven expression was very low, the maximal HRE-driven gene expression in both models was also considerably less efficient than CMV-driven expression. Since GFP fluorescence is dependent on oxygenation³⁷⁷ the levels of gene expression in this study may significantly underestimate the level of HRE driven expression. Further studies are underway to evaluate GFP expression by immunohistology and to use Lac Z reporter gene. While the present system is likely to minimise the possibility of toxicity resulting from inappropriate expression, the therapeutic and adverse effects of HRE-driven expression of an angiostatic protein such as sFlt-1 have yet to be evaluated.

Acknowledgements

I am very grateful to Katie Binley and Stuart Naylor of Oxford Biomedica for providing the HRE.GFP construct and to Mahesh De Alwis and Ajay Mistry for their help with AAV vector production.

6

Adeno-associated virus-mediated gene transfer to the canine retina

This chapter describes AAV-mediated gene transfer to the retina in an intermediate animal model. The surgical technique for delivery of vector suspension to the subretinal space of very young dogs is developed and AAV-mediated reporter gene expression is evaluated in a number of animals over an extended period. AAV-mediated transduction of cone photoreceptors is demonstrated, immune responses to the vector and transgene are characterised and the effect of subretinal vector delivery on retinal function is investigated by electroretinography.

6.1 Introduction

The results described in previous chapters have demonstrated that gene transfer of an angiostatic protein can inhibit angiogenesis in rodent models of retinal and choroidal neovascularisation and that angiostatic gene expression might be appropriately regulated by the incorporation of a tissue-responsive promoter. These findings, together with those of other groups, present a body of experimental evidence that supports a potentially valuable role for a gene transfer approach to ocular neovascular conditions in patients.

For the first human trials of ocular angiostatic gene transfer the clinical indications must be carefully chosen in order to optimise the likelihood of a significant therapeutic effect while minimizing the possibility of any adverse effect. Although the current treatment for proliferative diabetic retinopathy, panretinal photocoagulation, is associated with

predictable adverse effects this established approach can be effective in inducing the regression of retinal neovascularisation. While angiostatic gene transfer may offer a safer alternative in proliferative diabetic retinopathy, this novel approach might be most appropriately evaluated in the first instance in patients with neovascular AMD for whom no established effective treatment is currently available. One relatively simple approach in this condition would be to target retinal pigment epithelial cells and photoreceptors by a single subretinal administration of an AAV vector expressing the VEGF inhibitor sFlt-1. AAV vectors are particularly attractive vectors for this application because they mediate efficient sustained gene expression in these cell types.²³⁵ The pattern and kinetics of AAV-mediated reporter gene expression following subretinal vector delivery have been extensively described in rodents.^{270,275,378} While this work supports the future application of gene transfer techniques in clinical trials in human patients, significant differences between the rodent eye and the human eye present cause for concern. There have been few studies of AAV-mediated gene transfer in large animals,^{237,238,278,322} largely as a consequence of the substantial resources required for work of this nature, and experience of retinal gene transfer in large animals remains limited compared with rodent models. A prerequisite to the application of AAV-mediated retinal gene transfer in human patients is a thorough and rigorous evaluation of the efficacy and potential toxicity of the vector in an intermediate animal model.

The canine eye was chosen for this purpose for a number of reasons (Figure 6.1). Its larger size facilitates a more relevant surgical approach to the delivery of vector suspension to the subretinal space. Whereas in mice the small size of the globe and relatively large lens mean that only a trans-scleral, trans-choroidal approach is feasible, the larger size of the canine eye enables the use of standard vitreoretinal microsurgical techniques and a pars-plana approach which is the preferred technique in human patients. The mouse eye has very few cone photoreceptors that are scattered across the retina whereas the canine eye has a cone-rich visual streak that is more analogous to the human macula. The dog is more amenable to sophisticated assessments of visual function such as behavioural testing and its longevity enables the long-term evaluation of the effects of interventions.

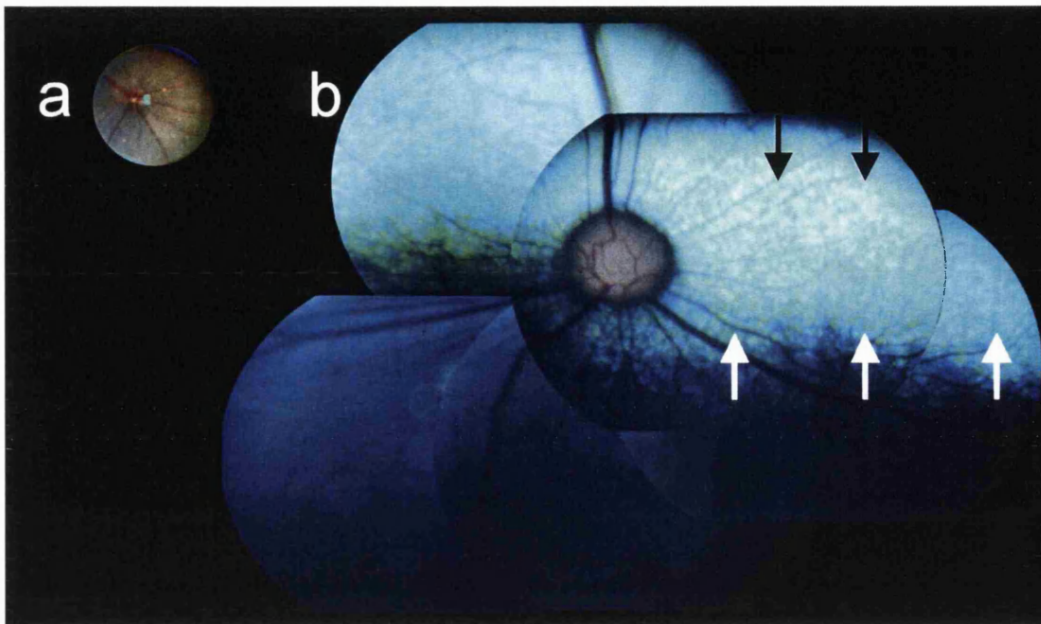


Figure 6.1 Fundus photographs of mouse and canine eyes

a, normal mouse eye; b, normal canine (beagle) eye. In the canine fundus photograph, the reflective tapetum is indicated by white arrows and the approximate location of the cone-rich macula-like visual streak is indicated by black arrows.

6.2 Vector production and delivery

6.2.1 Vector construction, virus production and determination of titre

Recombinant AAV (serotype 2) plasmids expressing green fluorescent protein (GFP) under the control of a cytomegalovirus (CMV) promoter (pHAV 5.5) were produced using the replicating amplicon system described previously²²⁸ and purified by elution through a heparin column. The titre of the purified viruses was determined by infectious

unit assay on 293 cells and by dot blot for DNA particles. The titre of injected vector suspension was 2×10^{10} infectious units/ml, 5×10^{11} particles/ml.

6.2.2 Surgery and anaesthesia

All animals were treated in a humane manner and were managed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Subretinal delivery of AAV.CMV.GFP was performed unilaterally in 8 normal dogs. Contralateral un-injected eyes were used as controls. Two animals were setter-beagle crosses aged 12 weeks and 6 animals were purebred beagles aged 6 weeks. The operated eye was pre-treated with topical prednisolone acetate 1% (Predforte) 4 times daily for 3 days pre-operatively. Pupillary dilatation was achieved with topical tropicamide 1% (Mydriacyl) and phenylephrine 2.5% 30 minutes pre-operatively. Anaesthesia was induced and maintained by inhalation of isoflurane in oxygen. Animals were intubated and ventilated using intermittent positive pressure and neuromuscular blockade was produced by intravenous vecuronium (Norcuron; Organon) 0.05-0.1 mg/kg. Sterile surgical technique was used throughout the procedure (Figure 6.2 a,b).

The eyelids and conjunctival sac were thoroughly cleaned with iodine solution and antibiotic prophylaxis provided using intravenous co-amoxiclav (Augmentin; SmithKline Beecham) 20 mg/kg at the start of surgery. The fundus was viewed by means of an operating microscope using a hand-held plano-concave contact lens with hydroxy-propyl-methyl-cellulose (HPMC) as a contact solution (Figure 6.2c). A pars-plana approach was used in all cases; a temporal sclerotomy was created approximately 3.5mm posterior to the corneoscleral limbus to accommodate a 33-gauge or 41-gauge subretinal cannula (MISS Ophthalmics, UK). In 6 animals a non-vitrectomy approach was used. In 2 animals a second sclerotomy was created for infusion of Hartman's solution (Baxter, Norfolk, UK) with 1:100 adrenaline and a core vitrectomy was performed. Since the relatively small palpebral aperture and prominent orbital margin in these animals offers only limited access to the sclera, the use of coaxial illumination from the operating

microscope was used in preference to a third scleral port for endoillumination. The vector suspension was drawn up through the subretinal cannula into a 1 ml syringe. The cannula was introduced through the scleral port, advanced through the vitreous cavity and used to create up to 3 retinotomies. Vector suspension was injected under manual control through the retinotomies into the subretinal space to raise retinal blebs (Figure 6.2d). This procedure was repeated at up to three sites in areas of tapetal and non-tapetal retina at the posterior pole around the optic disc. Total volumes of 200–400 μ l of virus suspension were delivered. Retinal vessels remained well perfused during and following the procedure. No attempt was made to close the retinotomies or to reattach the retina surgically. The sclerotomy ports were closed and subconjunctival injection of betamethasone was performed at the end of the procedure.

Post-operative analgesia was provided for 24 hours with buprenorphine (Vetergesic; Alstoe Ltd, UK) 0.01 mg/kg twice daily in addition to anti inflammatory treatment with carprofen (Rimadyl; Pfizer Ltd, UK) 2 mg/kg twice daily for 7 days and 1 mg twice daily for a further 7 days. All animals routinely received topical prednisolone 1 % and chloramphenicol 0.5 % 4 times daily, and topical atropine 1 % and ketorolac twice daily. The frequency of topical medication was gradually reduced and finally discontinued 6 weeks after the surgical procedure. Animals were examined clinically on the day following surgery and thereafter at weekly intervals. The anterior segment was examined for signs of intraocular inflammation and intraocular pressure was estimated by digital tonometry. The fundus was examined by indirect ophthalmoscopy to confirm retinal re-attachment and to identify any signs of adverse effects.

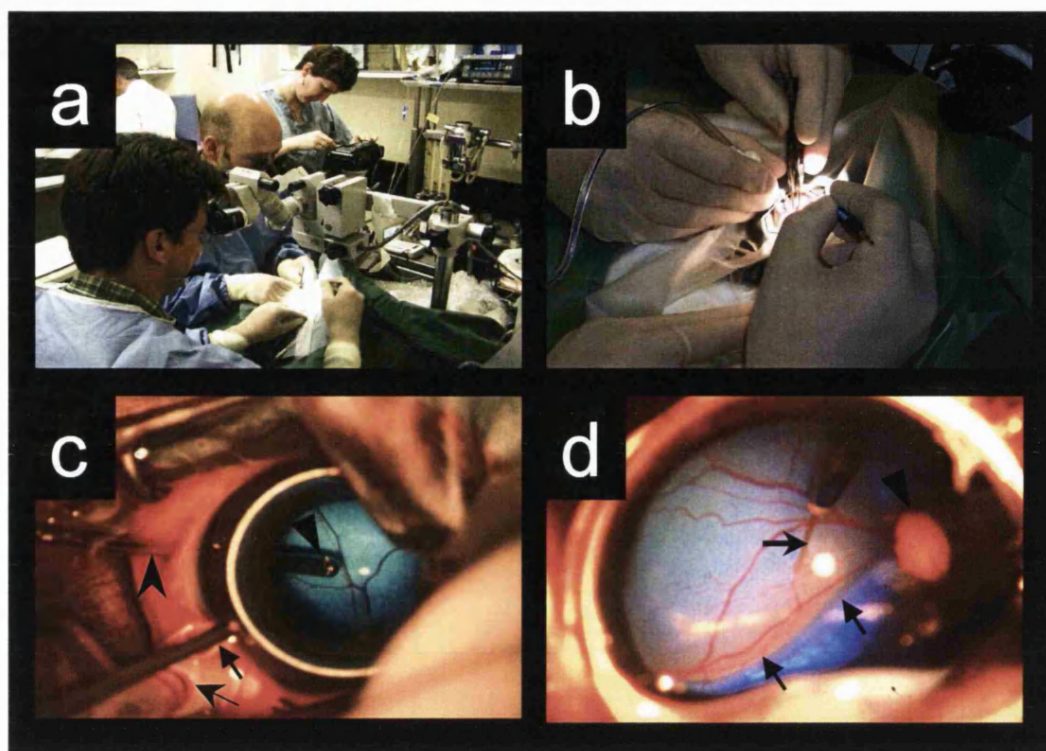


Figure 6.2 Surgical technique for subretinal delivery of vector suspension in dogs

a, operating theatre during procedure; b, surgical field; c, surgeon's view of operating field showing irrigating contact lens (arrow) and irrigation cannula (open arrow) sutured in place in a scleral port. The shaft (open arrowhead) and tip (arrowhead) of the vitreous cutter passed through a second scleral port are also indicated; d, surgeons' view of the fundus during subretinal delivery of vector suspension. The tip of the 41 gauge subretinal cannula (open arrow) is used to create a retinotomy. Injection of vector suspension into the potential subretinal space raises a 'bleb' of neurosensory retina, the inferior border of which is indicated (arrows). The optic nerve head is also indicated (arrowhead).

Subretinal delivery of AAV was performed unilaterally in 8 normal dogs. Contralateral un-operated eyes were used as controls. Details of individual animals are summarised in Table 6.1. Optimal retinal transduction was achieved by first performing a core vitrectomy before raising retinal blebs of vector suspension using a 41-gauge subretinal

cannula to create multiple retinotomies. Total volumes of up to 400 μ l of virus suspension were injected through up to 3 retinotomies in each case. Using this technique it was possible to create blebs extending across the greater part of the posterior pole, involving up to an estimated 20 % to 30 % of the total retinal area. The surgical procedures were uncomplicated except for an inadvertent posterior retinal tear in one case. In all cases the retinal bleb flattened spontaneously within 24 hours of the procedure and the retina remained attached for the duration of the study.

Table 6.1 Summary of experimental data

Case number	Breed	Age at	Pars-plana	Onset of	ERG	Intraocular	Histology	AAV		GFP	
		procedure	vitrectomy	GFP	performed	inflammation	timepoint	Serology(OD)	Serology(OD)	pre-op	post-op
		(weeks)	performed	expression		(weeks to	(weeks)			pre-op	post-op
				(weeks)		onset)					
1	setter/beagle	16	no	3	no	none	NA	0.0	1.5	0.2	2.0
2	setter/beagle	28	no	3	no	4	6	0.0	3.3	1.0	3.4
3	beagle	6	no	2	yes	none	NA	0.8	3.6	1.1	6.9
4	beagle	6	no	3	yes	none	NA	1.0	1.5	1.3	2.2
5	beagle	6	no	2	no	5	8	2.3	3.7	3.2	22.7
6	beagle	6	no	2	no	none	8	0.7	1.1	0.9	1.4
7	beagle	8	yes	3	yes	none	9	1.0	U	1.3	U
8	beagle	8	yes	3	yes	6	9	2.3	0.6	1.5	17.5

NA, histology not yet performed; OD Optical density (arbitrary units); U, sample unavailable for analysis

6.3 Evaluation of reporter gene expression *in vivo*

GFP reporter gene expression was determined by fluorescence funduscopy *in-vivo*. Fundus fluorescence photography was performed at weekly intervals following vector delivery using a Kowa Genesis fundus camera (Keeler, UK) with appropriate excitation

and emission filters. Images were digitalised and fundus montages created using Adobe Photoshop.

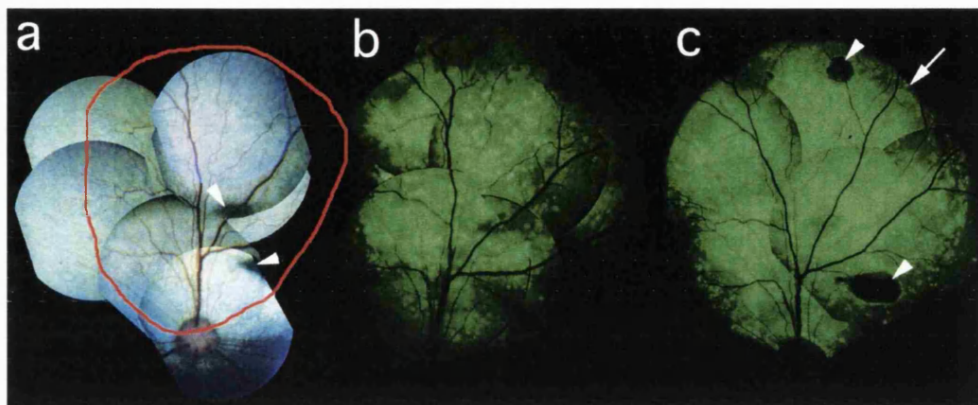


Figure 6.3 Fundus photograph and in-vivo fundus GFP fluorescence following subretinal delivery of AAV.CMV.GFP

Colour fundus photograph 6 weeks following vector delivery to tapetal retina at a site superior to the optic disc. The approximate area of subretinal vector delivery and subsequent GFP expression is outlined in red. No effect on fundus appearance is observed except for focal loss of tapetal reflectivity at retinotomy sites (white arrowheads). (b) In-vivo fundus fluorescence photograph 6 weeks following vector delivery. Efficient GFP fluorescence is evident across the area of the original retinal bleb. (c) In-vivo fundus fluorescence photograph 12 months following vector delivery. Sustained GFP expression is evident. The area of GFP fluorescence is unchanged and has a well-defined border (white arrow). Focal areas devoid of fluorescence are evident at retinotomy sites (white arrowheads).

GFP expression was evident by fluorescence funduscopy in all cases. Expression was first observed at 2 to 4 weeks postoperatively, increasing in intensity until 6 to 8 weeks when the intensity of expression reached a stable maximum (Figure 6.3). The area of GFP fluorescence was well -defined, confined clinically to the site of the original retinal bleb(s) and has been sustained for up to 18 months at the present time. The intensity of GFP expression was similar in areas of tapetal and non-tapetal fundus. Small discrete

areas of loss of the tapetal reflex were evident at retinotomy sites and GFP fluorescence at these sites was absent. No fluorescence was detected by funduscopy in un-operated eyes.

6.4 Evaluation of retinal function by electroretinography

6.4.1 Effect of subretinal AAV delivery on electroretinogram

The effect of local subretinal delivery of AAV.CMV.*GFP* on global retinal function in four animals was evaluated using full-field flash photopic and scotopic electroretinography (ERG). ERGs were recorded in a standardised fashion at 4 weeks, 9 months, 12 months and 15 months after vector delivery. All animals were dark-adapted for 60 minutes. All procedures for recording were performed under dim red light. Animals were sedated by intramuscular injection of medetomidine 10 µg/kg and butorphanol 0.4 mg/kg. The pupils were dilated using topical phenylephrine 2.5% and tropicamide 1%. A single drop of 2% HPMC was placed on each cornea to maintain epithelial hydration. ERGs were recorded using a commercially available instrument (Toennies Multiliner Vision, Jaeger/Toennies) after placing corneal contact lens electrodes (Type Henkes, Jaeger/Toennies) and midline sub-dermal reference and ground electrodes. Bandpass filter cut-off frequencies were 1 Hz and 300 Hz for all measurements. Ganzfeld ERG responses were recorded simultaneously from the subretinally injected right retina and the uninjected left retina to control for inter-animal and test-retest variance. Care was taken to ensure that electrode impedances were minimal (approximately 5 kohm) and symmetrical between the two eyes. Single flash recordings were obtained at light intensities increasing from 0.1 mcDs/m² to 3000 mcDs/m². Ten responses per intensity level were averaged with an inter-stimulus interval of 5s (0.1, 1, 10 and 100 mcDs/m²) or 5 responses per intensity with a 17s interval (1000 and 3000 mcDs/m²). These intervals were chosen in order to minimise the dark-adapted flash effect. After electroretinography was completed the medetomidine was reversed using atipamezole 50 µg/kg. The data were analysed using the Toennies Multiliner

Vision program. For each animal the b-wave amplitudes at 100 mcads/m² and 1000 mcads/m² were used for statistical analysis. The b-wave values (a-wave trough to b-wave peak) of the treated right eyes were compared with those of the untreated left control eyes and the mean difference for all animals was calculated.

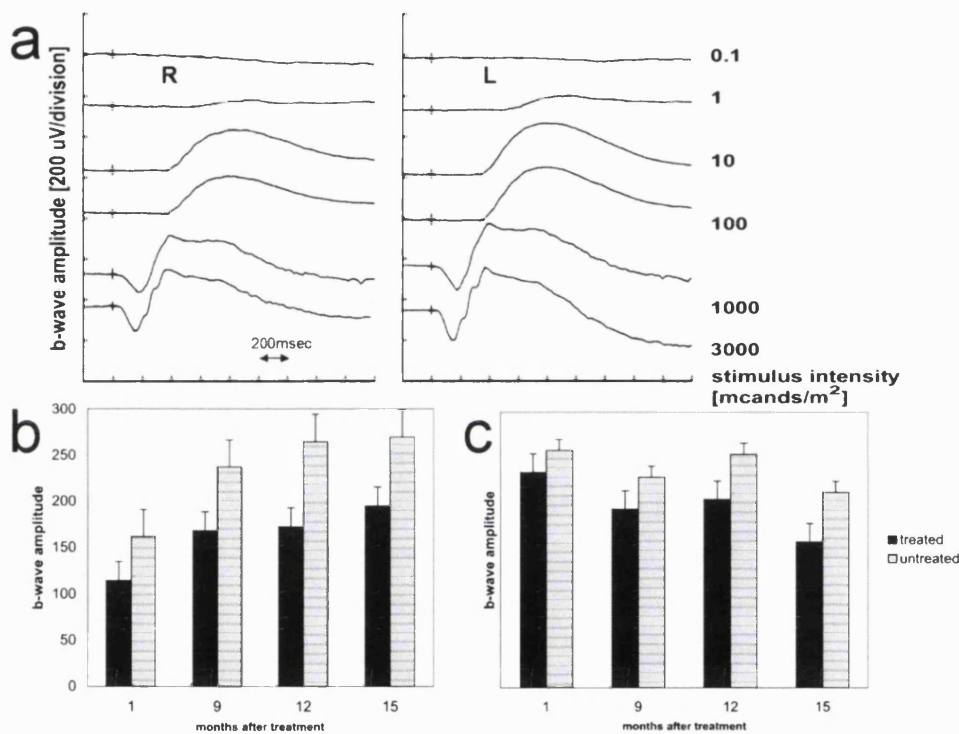


Figure 6.4 Electrophysiology 8 weeks following subretinal delivery of AAV.CMV.GFP in normal 6-week-old beagles

(a) Representative simultaneous recordings from the injected (R) and uninjected (L) eye 4 weeks following vector delivery. Amplitude of responses (200 μ V per division) following a series of increasingly intense full-field flash stimuli were recorded. The ERG responses showed a normal pattern with some attenuation of b-wave amplitude in the injected eye compared to the uninjected eye. (b,c) ERG b-wave amplitudes (μ V) following gene delivery (mean \pm SD). (b) responses to 100 mcads/m² stimulus, (c) responses to 1000 mcads/m² stimulus.

The effect of local subretinal delivery of AAV.CMV.GFP on global retinal function was evaluated in four animals by eliciting ERG responses to a series of increasing stimulus intensities (Figure 6.4). In normal animals, increasing stimulus intensity results in responses of increasing amplitude and shortened latency of the ERG b-wave. In this experiment, normal patterns of responses to increasing stimuli, in terms of both the latency and amplitude of the b-wave were elicited in both eyes of all animals studied. A variable reduction in b-wave amplitude, however, was observed following local subretinal delivery of AAV.CMV.GFP compared to untreated contra-lateral eyes. (Figure 6.4a) In order to summarise ERG responses in this group of animals, mean b-wave amplitudes were calculated for each of two stimulus intensities (100 mcands/m² and 1000 mcands/m²). The b-wave amplitude following subretinal vector delivery was consistently reduced by approximately 30% for 100 mcands/m² stimuli (Figure 6.4b) and by approximately 20% for 1000 mcands/m² stimuli (Figure 6.4c).

6.4.2 Effect of subretinal PBS delivery on retinal function

In order to determine the extent to which retinal detachment alone contributed to the observed functional attenuation following subretinal delivery of AAV suspension, further studies were performed in which PBS was delivered to the subretinal space of normal dogs. Pars plana vitrectomies were performed in the right eyes of four normal beagle dogs, 6 weeks of age, as described above. Trans-retinal injections of sterile PBS were performed at up to 3 sites using a total volume of approximately 400 µl of PBS in each eye to raise blebs of neurosensory retina involving up to an estimated 30 % of the total retinal area. The surgical procedures were uncomplicated. In all cases the retinal blebs flattened spontaneously within 24 hours of the procedure and the retina remained attached for the duration of the study. The effect of local subretinal delivery of PBS on global retinal function was evaluated using full-field flash photopic and scotopic electroretinography (ERG) as described above at 10 weeks and 20 weeks following the surgical procedure.

Normal patterns of responses to increasing stimuli, in terms of both the latency and amplitude of the b-wave were elicited in both eyes of all four animals studied. A reduction in b-wave amplitude was observed following local subretinal delivery of PBS only at the 10 week time point at the 100 mcands/m² stimulus intensity ($p=0.02$) (Figure 6.5b). There was no significant difference at the 1000 mcands/m² stimulus intensity at this time point. After 20 weeks b-wave amplitudes were symmetrical at both stimulus intensities (Figure 6.5a, 6.5c).

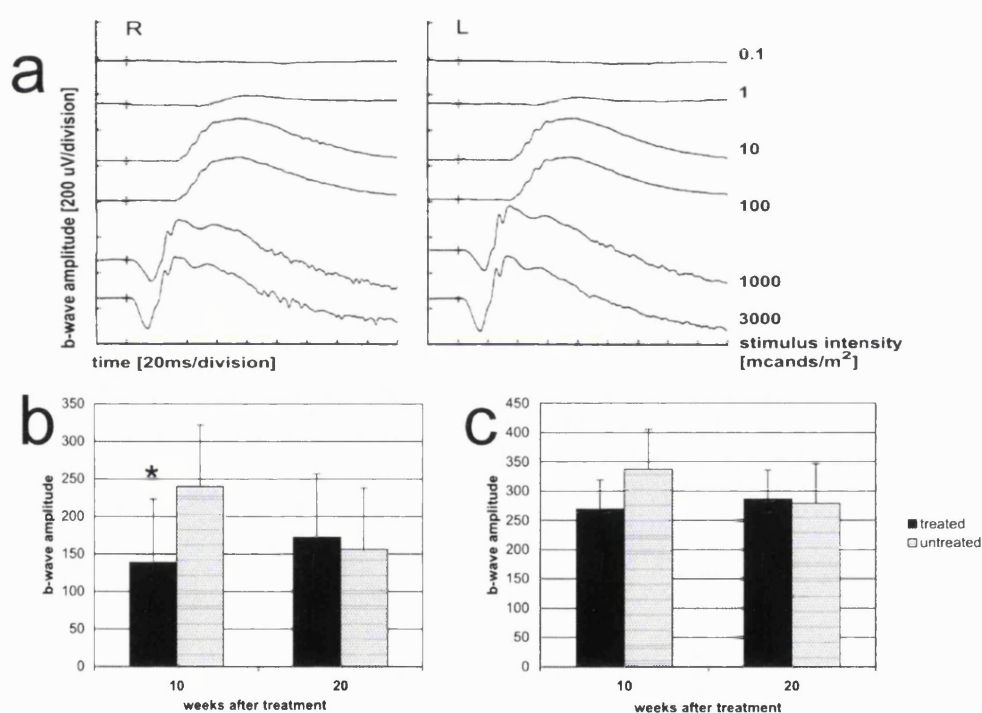


Figure 6.5 Electrophysiology 10 and 20 weeks following subretinal delivery of PBS in normal 6-week-old beagles

(a) Representative simultaneous recordings from the injected (R) and uninjected (L) eye 20 weeks following PBS delivery. Amplitude of responses (200 μ V per division) following a series of increasingly intense full-field flash stimuli were recorded (20ms/division). The ERG responses showed a normal pattern with symmetrical b-wave amplitudes. (b,c) ERG b-wave amplitudes (μ V) 10 weeks and 20 weeks following PBS delivery (mean \pm SD). (b) responses to 100 mcands/m² stimulus (* $p = 0.02$), (c) responses to 1000 mcands/m² stimulus.

6.5 Histological examination

For histological assessment of reporter gene expression 5 dogs were humanely killed by intravenous injection of pentobarbitone 6 to 8 weeks following vector delivery. In animals where signs of intraocular inflammation were clinically apparent, vitreous and aqueous samples were cultured to investigate the possibility of infective endophthalmitis. Following enucleation a full-thickness incision was made at the limbus and globes were immersion-fixed in freshly prepared 4% paraformaldehyde in PBS for 1 hour. The eyecup was divided from the anterior segment and fixed for a further 24 hours. Cryosections for GFP fluorescence and wax sections for haematoxylin and eosin were prepared as previously described (Chapter 2).

6.5.1 Reporter gene expression

Fluorescence microscopy of cryosections from the area of the retinal bleb demonstrated efficient transduction of photoreceptor cells and retinal pigment epithelial cells 6 weeks following subretinal vector delivery (Figure 6.6). The transduction rate of rod photoreceptors in the area of the retinal bleb was approximately 35 %. In the retinal pigment epithelium efficient expression of GFP was evident as a confluent band in the area of the retinal bleb, consistent with a transduction rate of greater than 50 %. Expression of GFP in photoreceptors and RPE cells was not evident beyond the area of subretinal vector delivery. No GFP fluorescence was observed in un-operated eyes.

GFP fluorescence was evident in cells of the ganglion cell layer scattered across the posterior pole and in nerve fibres at the optic nerve head (Figure 6.7a,b). GFP expression was also observed in cells of the iris and ciliary epithelium (Fig 6.7c) though not in the lens, cornea or trabecular meshwork. Extra-retinal expression was most prominent in eyes in which the subretinal vector delivery had been preceded by core vitrectomy.

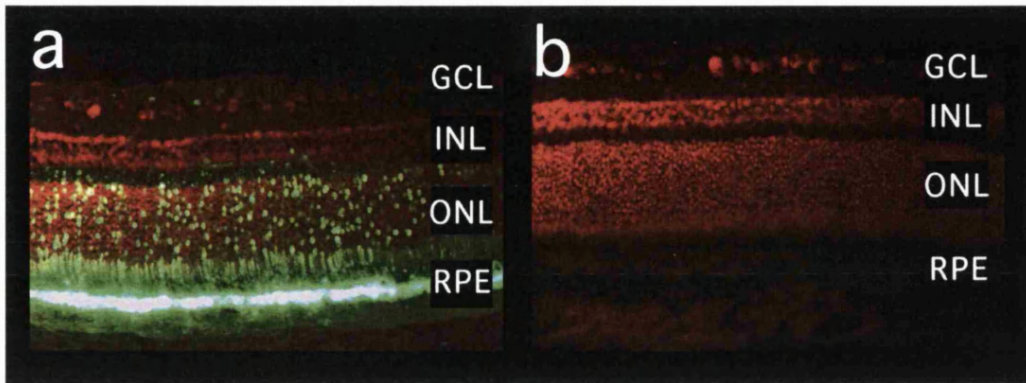


Figure 6.6 Fluorescence micrographs of retinal cryosections 6 weeks following subretinal delivery of AAV.CMV.GFP vector to right eye

(a) Section of retina from site of vector delivery at posterior pole of right eye. GFP fluorescence is evident in photoreceptor cells in the outer nuclear layer (ONL) and at the level of the retinal pigment epithelium (RPE). Only occasional cells in the ganglion cell layer (GCL) and inner nuclear layer demonstrate GFP expression. (b) Section of retina from an equivalent area of the contra-lateral uninjected control eye. No GFP fluorescence is evident.

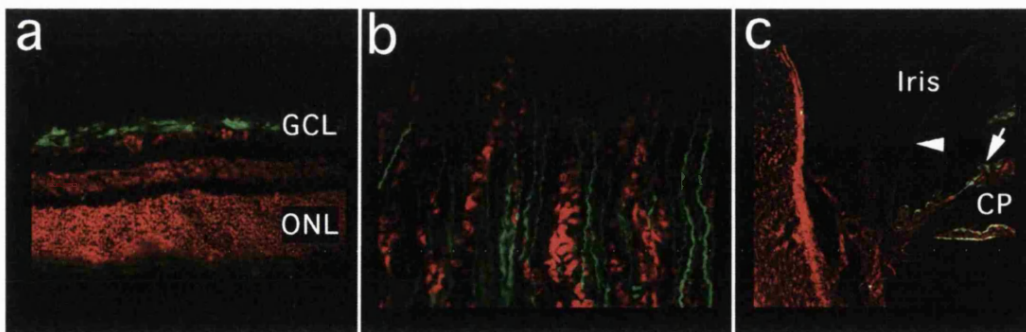


Figure 6.7 Fluorescence micrographs of cryosections 6 weeks following subretinal delivery of AAV.CMV.GFP

(a) Section of retina from site distal to area of subretinal bleb showing GFP expression in cells of ganglion cell layer (GCL). (b) Section through optic nerve showing GFP expression in nerve fibre axons. (c) Section of anterior segment showing iris and ciliary processes (CP). GFP fluorescence is observed in ciliary epithelium (arrow) and in iris epithelium (arrowhead).

6.5.2 AAV-mediated transduction of cone photoreceptors

In addition to their potential therapeutic value for the delivery of angiostatic proteins in neovascular disorders, AAV vectors have long been seen as potentially valuable for the targeting of photoreceptor cells in gene therapy strategies for inherited retinal degenerations²⁷⁴. Although the ability of AAV vectors to efficiently transduce rod photoreceptor cells is well established, AAV-mediated transduction of cone photoreceptor cells has not previously been demonstrated.²³⁷ This question is of clinical importance because a significant number of inherited retinal degenerations are cone-based.³⁷⁹

In order to determine whether cone photoreceptors are transduced by AAV, cryosections of canine retina following delivery of AAV.CMV.GFP vector were stained to identify cone photoreceptors as described in Chapter 2. Briefly, sections were incubated overnight at 4°C with biotinylated peanut agglutinin, a lectin that specifically binds to the glycocalyx of cone photoreceptor cells,³⁸⁰ followed by Alexa594-conjugated streptavidin for 2 hours at room temperature. The sections were washed before mounting in fluorescence mounting medium for examination by confocal microscopy. The transduction efficiency of cone photoreceptors was determined by counting the number of GFP positive and negative lectin-stained cells in 8 randomly selected high-power fields (146 µm wide) of the transduced area.

Histochemical staining by peanut agglutinin resulted in bright staining of the glycocalyx of the inner and outer segments of the cone photoreceptors. (Figure 6.8a) A fainter, but clear staining was present around the cell body of the cone photoreceptors. The cell bodies of cones are located at the border between the outer nuclear layer and the inner segment area³⁸¹ confirming the specificity of our staining. Although most of the GFP positive photoreceptors were rods, in a minority of cells GFP co-localised precisely with peanut agglutinin staining of cones. In these cells the GFP was present in the cell bodies and the inner segments and was bordered by the peanut agglutinin staining of the plasma membrane. It is estimated that 5 % to 10 % of the cone photoreceptors expressed GFP.

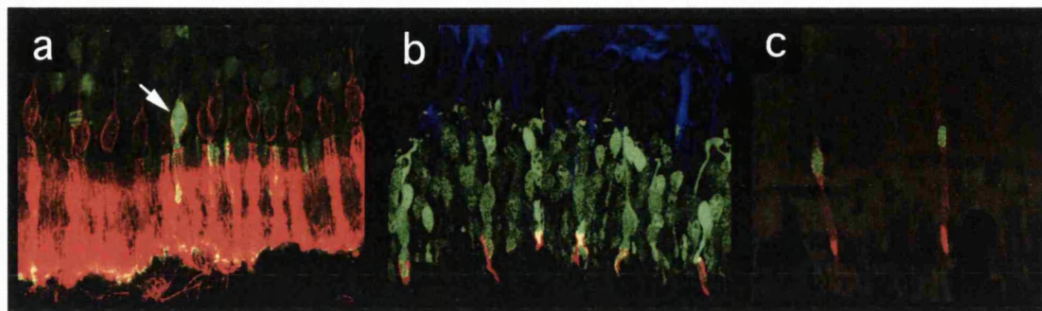


Figure 6.8 AAV-mediated transduction of cone photoreceptors; scanning laser confocal micrographs of sections of outer retina

(a) Canine retina 6 weeks following subretinal delivery of AAV.CMV.GFP. GFP expression co-localises with a peanut agglutinin-stained cone photoreceptor (arrow). (b) Feline retina 8 weeks following subretinal delivery of AAV.CMV.GFP. Intermediate filaments labelled with anti-GFAP (blue) in Müller cells extend into the outer nuclear layer. GFP expression is evident in photoreceptor cells and co-localises with a peanut agglutinin-stained cone photoreceptor sheaths (red). (c) Feline retina 8 weeks following subretinal delivery of AAV.CMV.GFP. Expression of GFP is clearly demonstrated in the cell bodies of two cones specifically labelled with anti-M/L cone opsin (red).

The ability of AAV vectors to transduce cone photoreceptors was further investigated in a second cone-rich intermediate animal model- the feline retina. Two normal adult female cats (aged 8 months) underwent pars plana vitrectomy and subretinal delivery of AAV.CMV.GFP vector suspension using a technique similar to that described above. The animals were humanely killed 8 weeks postoperatively by intravenous injection of pentobarbitone and their eyes were fixed in 4% paraformaldehyde. Areas of transduced retina were agarose-embedded and 100 μ m thick sections were stained to identify cone photoreceptors using either biotinylated peanut agglutinin or anti-medium to long wavelength (M/L) cone opsin. Confocal microscopy of normal feline retina in the region of subretinal vector delivery demonstrated efficient GFP expression in photoreceptor cells. In a proportion of photoreceptors GFP expression was found to co-localise with cone-specific staining by peanut agglutinin (Fig 6.8b). Furthermore, GFP expression was

found to co-localise with immuno-staining of cones by an anti-M/L cone opsin antibody (Fig 6.8c). Vector delivery and histological analysis in cats were performed by Charanjit Sethi.

6.6 Serology and immune responses

Antibody responses to AAV capsid proteins and to GFP protein were determined by sandwich ELISA on serum samples taken pre-operatively and after 8 weeks following vector delivery (Table 6.1) as described in Chapter 2. No significant serum antibody response to AAV capsid proteins was detected following subretinal vector delivery in any animal. Significant antibody responses to GFP protein were detected, however, in 2 animals following vector delivery (animals #5 and #8).

Three of the 8 dogs that received subretinal delivery of AAV.CMV.GFP developed a delayed-onset intraocular inflammatory response 4 to 6 weeks following (Table 6.1). These animals developed progressive conjunctival injection, anterior chamber activity (without hypopyon), posterior synechiae and vitreous haze. Microscopy of haematoxylin and eosin-stained sections demonstrated a diffuse, non-granulomatous chronic inflammatory cell infiltrate involving the retina with focal extensions into the choroid. There was peri-vascular inflammation along the course of the central retinal vessels and involvement of the fibroglial septae of the optic nerve. The extent of inflammatory changes was most marked at the area of subretinal vector delivery but was also evident to lesser degree in other parts of the retina. Retinal GFP expression was observed histologically in inflamed eyes though the level of expression appeared lower than that in un-inflamed eyes. Samples of aqueous and vitreous revealed no organisms on gram-stain and no growth on culture. In 2 of the 3 animals that developed intraocular inflammation there were significant levels of GFP-specific antibodies. Animals without clinical signs of intraocular inflammation did not develop significant serological responses to GFP protein. Treatment of the intraocular inflammation in one animal by systemic and intensive topical corticosteroids resulted in a dramatic clinical improvement over the

course of a 2-week period with resolution of anterior chamber flare and vitreous haze. The remaining 2 animals were sacrificed for analysis soon after the diagnosis of intraocular inflammation and in these cases the effect of immunosuppressive therapy was not investigated. Clinical signs of uveitis were not observed in any of the further four animals that received subretinal injections of PBS nor in un-operated fellow eyes during the study period.

6.7 Discussion

The results of this study demonstrate significant differences between the rodent eye and the canine eye with respect to AAV-mediated retinal gene transfer and highlight the importance of extending the evaluation of such vectors from rodents to more clinically relevant animal models before embarking on applications in patients.

6.7.1 Surgical technique for subretinal delivery of vector suspension

Conventional vitreoretinal microsurgical techniques facilitated the effective delivery of vector suspension to the subretinal space in these animals. Subretinal vector delivery was possible using a non-vitrectomy trans-vitreous technique but a limited pars-plana vitrectomy offered a greater degree of surgical control and facilitated the delivery of larger volumes of vector suspension. Manually-controlled injection of vector suspension using a 41-gauge cannula through multiple retinotomies to create retinal ‘blebs’ that became confluent was found to be more effective than a single bleb in achieving vector delivery to a large area of the retina. The vector was delivered in animals at 6 weeks of age because in many inherited retinal disorders progressive degeneration occurs at an early age and it is anticipated that gene-replacement strategies for these conditions are likely to be most effective when established at an early stage in the disease process.²⁷⁵

6.7.2 AAV-mediated reporter gene expression

The results of these experiments demonstrate that, in dogs, reporter gene expression in photoreceptors and RPE cells does not extend significantly beyond the original area of vector delivery even in the long term. This finding is consistent with previous reports of subretinal vector delivery in large animals using both AAV^{237,278} and lentiviral²⁶² systems and has important implications for clinical applications. Effective gene transfer to a given area of retina by subretinal delivery of this vector is likely to require surgical detachment of the entire area. The control of retinal transduction by surgical delivery, however, offers a valuable means to minimise adverse effects of gene transfer on non-target retina.

Following subretinal delivery of AAV.CMV.GFP in mice, transgene expression is restricted to cells of the outer retina and is not observed in cells of the inner retina or anterior segment. In contrast, in dogs we observed significant GFP expression in ganglion cells scattered across the posterior pole and in optic nerve fibres. GFP fluorescence was also observed in cells of the iris and ciliary epithelia, though not in the lens, cornea or trabecular meshwork. It is most likely that the difference in patterns of tissue transduction between mice and dogs is the result of refluxed vector suspension from the subretinal space into the vitreous cavity through the retinotomy in dogs. This effect appeared to be more marked following vitrectomy, possibly because of the tendency of an intact vitreous gel to restrict diffusion of vector suspension. Evidence of transduction of ganglion cells (though not anterior segment tissues) following intravitreal injection of AAV vectors in dogs in a previous study supports the role of this mechanism.³²² In mice the small size of the globe means that a transcleral, transchoroidal approach is conventionally used for subretinal vector delivery. Since a retinotomy is not normally created, the passage of vector suspension from the subretinal space into the vitreous and anterior chamber does not tend to occur. Following subretinal delivery in primates only rare GFP positive ganglion cells, confined to the point of needle entry, have been observed and none in the optic nerve or anterior segment.²³⁷ For clinical applications in patients, however, the

possibility of vector reflux into the vitreous cavity cannot be excluded and for this reason the restriction of gene expression to target cells by means of tissue-specific promoters may be required to prevent adverse effects resulting from inappropriate expression of therapeutic genes in non-target cells.

6.7.3 AAV-mediated transduction of cone photoreceptor cells

Following subretinal delivery in dogs, AAV-2 vectors mediated efficient GFP expression in photoreceptors and in retinal pigment epithelial cells. Previous studies in primates have suggested that AAV-mediated photoreceptor transduction is specific to rods.²³⁷ The results of the present experiments conclusively demonstrate for the first time that AAV vectors are able to transduce cone photoreceptors in addition to rods and this observation is confirmed in a second cone-rich intermediate animal model- the feline retina. This finding is of clinical importance because inherited retinal degenerations are the commonest causes of inherited visual loss, the majority are currently untreatable, and a significant number of these result from mutations in genes expressed in cones.³⁷⁹ In areas of retinal transduction in the canine eye approximately 5 to 10 % of the cone photoreceptors expressed GFP. This proportion is substantially smaller than that of rods expressing GFP in the same area and suggests that cone photoreceptor cells may be less efficiently transduced by AAV than rods. Thus the apparent absence of AAV-mediated cone transduction in rodents may be a result of a relative inefficiency of transduction by AAV vectors as well as the difficulty in identifying transduced cone photoreceptor cells in a retina that is cone-poor. The ability of AAV vectors to efficiently transduce cones significantly extends their range of potential applications to include cone-based retinal degenerations.

6.7.4 Immune responses following subretinal delivery of AAV.CMV.GFP

Clinical and histological evidence of an intraocular inflammatory response was observed in 3 of 8 animals following subretinal delivery of AAV.CMV.GFP. In our experience marked inflammatory responses are not observed following subretinal delivery of this vector in mice. Neither was ocular inflammation observed following vector delivery in the 2 cats described in this study. Possible causes for the inflammation in the dogs include sterile inflammatory responses to the surgical procedure, subacute bacterial endophthalmitis and immune responses to the viral vector or to the expressed protein GFP. The histological appearances were consistent with a non-specific inflammatory reaction to the surgical procedure or with an immune-mediated response. Dogs are particularly liable to develop aggressive inflammatory responses following intraocular surgery, often in association with a fibrinous anterior uveitis. This type of response, however, seems an unlikely explanation for the inflammation observed in these animals as clinical signs of inflammation were not apparent until several weeks after vector delivery. In addition, uveitis was not observed in any of the further four animals that received subretinal injections of PBS.

Many of the features of the inflammatory responses are consistent with an immune response to expressed GFP protein. The onset of clinical signs of inflammation coincided with the establishment of maximal GFP expression and the histological changes were more marked in areas of transduced retina than in retina elsewhere in the same eye. Significant serum antibody responses to GFP protein were detected in 2 of the 3 animals that developed inflammatory responses and in none of the unaffected animals tested. A serum antibody response to GFP in a dog following subretinal delivery of AAV.CMV.GFP has been previously described³²² though this was not apparently associated with intraocular inflammation.

The intraocular inflammation is not likely to be the result of antibody responses to vector-specific proteins. AAV is of low immunogenicity and in previous studies, even where significant serum antibody responses to viral capsid proteins have been detected following subretinal delivery of AAV.CMV.*GFP* in nonhuman primates²³⁷ or intravitreal delivery in dogs,³²² there has been no clinical or histological evidence of inflammatory response. In the present study no significant serum antibody response to capsid proteins was detected, and the onset of inflammation was delayed until some 4 to 6 weeks following vector delivery. Subacute endophthalmitis was excluded on the basis of negative microbiological investigations and the atypical histological appearances. Intraocular inflammation in the animal treated with intensive topical and systemic steroids responded very favourably without concomitant antibiotic cover suggesting that the primary cause of the inflammation was not microbial.

The induction of an immune response directed against expressed GFP reporter gene is not directly relevant to clinical applications in which other, therapeutic proteins are expressed. The possibility that similar immune responses might be directed against such expressed therapeutic proteins, however, is an important concern and its significance is twofold. Firstly, such a response may neutralise the therapeutic effect of the expressed protein and secondly, the induction of sustained intraocular inflammation might cause significant adverse effects resulting in visual loss. In the present study the majority of animals tolerated efficient retinal GFP expression in the long-term with no evidence of intraocular inflammation. The reason that only a minority of animals developed immune responses in this study is not clear. One may speculate that the intensity and duration of immune responses to an expressed protein might depend on individual predispositions, the degree of compromise of the blood retinal barrier and the nature of the expressed protein, for example its similarity to endogenously expressed proteins. The favourable response to prompt immunosuppressive treatment in this study suggests that such inflammatory reactions might be effectively controlled in the clinical context. These findings, however, highlight the possibility of transgene-induced immune responses that should be carefully considered in future pre-clinical and clinical studies of retinal gene transfer.

6.7.5 Evaluation of retinal function following subretinal delivery of AAV.CMV.GFP

Few studies have evaluated the functional effects of AAV vector delivery to the retina in normal animals. In the experiments described in this thesis, electroretinography demonstrated a modest but consistent and sustained reduction in global retinal function following subretinal delivery of AAV.CMV.GFP even in the absence of clinical signs of intraocular inflammation. In contrast, subretinal delivery of PBS resulted in a transient attenuation in the ERG response at 10 weeks that had fully recovered by 20 weeks postoperatively. These results suggest that brief surgically-induced detachment of the posterior retina does not have significant consequences for long-term global retinal function in the dog but that injection of AAV.CMV.GFP can result in a modest sustained attenuation in retinal function. Short-term surgically-induced retinal detachment in the rabbit also results in only transient reduction in ERG responses.³⁸² Although macular translocation surgery in humans with choroidal neovascularisation can result in sustained ERG changes,^{383,384} this procedure involves detaching and manipulating the neurosensory retina in a technique that is significantly more complex than a simple localised detachment by transretinal injection.

The attenuation of retinal function following subretinal vector delivery may be a consequence of toxicity of the carrier medium, AAV-vector or expressed GFP. Although similar work in nonhuman primates demonstrated no electroretinographic evidence of toxicity following subretinal delivery of AAV.CMV.GFP, smaller volumes of vector suspension (50-100µl) were used and subtle changes in ERGs may have been undetected.²³⁷ In the present study the likelihood of impurities in the AAV vector preparation causing retinal toxicity is small since the purification process involved repeated passages through caesium chloride, but this possibility is being addressed by a review of the preparation protocols. As discussed above, AAV is known to be of low pathogenicity and did not induce significant antibody responses in the animals in this study. The possibility of that GFP may be toxic, however, is an important concern. There is accumulating evidence that GFP can be cytotoxic³⁸⁵ and induces apoptosis of neuronal

cells.³⁸⁶ GFP expression in RPE cells is associated with light-dependent toxicity *in vitro*³⁸⁷ and morphological changes *in vivo*.³⁸⁸ Following subretinal injection of high-titre AAV.CMV.*GFP* in dogs in this study, highly efficient GFP expression was evident both on fundus fluorescence photography and on fluorescence microscopy. The possibility that GFP toxicity may be responsible for the sustained functional attenuation following subretinal injection of AAV.CMV.*GFP* will be investigated in further studies using an alternative reporter gene such as Lac Z. The development of non-toxic fluorescent reporter genes will offer a valuable alternative to GFP for use in future experiments.

6.8 Conclusion

These experiments have identified a number of important differences with respect to retinal gene transfer between dogs and mice. In dogs AAV-mediated gene expression in photoreceptors and RPE cells does not extend beyond the original area of vector delivery. Significant expression can occur, however, in the inner retina, optic nerve and ciliary epithelium, presumably as a result of vector reflux through the retinotomy. The ability of AAV to transduce cone photoreceptors in addition to rods is demonstrated following vector delivery to cone-rich areas of both the canine and the feline retina. Subretinal delivery of AAV vector in dogs was associated in these experiments with a variable reduction in global retinal function as assessed by electroretinography that may be a consequence of GFP toxicity. Intraocular inflammation, possibly the result of an immune response to expressed GFP protein, was observed in a minority of animals but in the majority of cases AAV-mediated expression was well tolerated over a long period.

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7

Discussion

In this chapter the results of the experiments described in previous chapters are briefly discussed in the wider context. The rationale for a clinical trial of angiostatic gene transfer in neovascular AMD is presented and a proposal for such a trial described. Finally, future developments that are required to facilitate the optimisation of angiostatic gene transfer approaches are discussed.

7.1 Conclusions

7.1.1 Time-courses of vector-mediated expression in experimental retinal and choroidal neovascularisation

The results of experiments described in the preceding chapters offer proof of principle that local gene transfer of angiostatic proteins can arrest the progression of angiogenesis in different models of ocular neovascularisation. Murine models of ischaemia-induced retinal neovascularisation and laser-induced choroidal neovascularisation were established and optimised in order to evaluate the transduction patterns of viral vectors. Having previously characterised gene expression mediated by Ad, AAV and lentiviral vectors in the eyes of normal animals, the targeting, efficacy and kinetics of transgene expression were evaluated in the contexts of retinal and choroidal angiogenesis. Since the angiogenic responses in both these models are self-limiting and relatively short-lived, in order to establish transgene expression during the peak of angiogenic drive vectors were delivered prior to its onset. As Ad and lentiviral vectors mediate established expression within a few days of vector delivery^{213,261} these vectors could be effectively delivered only a few days in advance of the neovascular response. Indeed lentivirus-

mediated expression of an angiostatic factor may be effective when the vector is delivered subsequent to laser-induction of choroidal neovascularisation, a sequence of events that more closely reflects the clinical scenario, and this possibility is the subject of ongoing studies. Gene expression by AAV-2 vectors, in contrast, does not reach maximal levels for 4-6 weeks²⁴³ and for this reason these were delivered as early as possible; in order to achieve established expression in the model of retinal neovascularisation (which can be induced only in very young animals) AAV vectors were delivered at p2 in anticipation of maximal angiogenesis at p14-p16. Although this resulted in a significant angiostatic effect even 2 weeks later, the efficiency of AAV-2-mediated expression at this point represents only a fraction of the potential maximum that might be achieved after a further 2-4 weeks. Alterations in genome length and AAV capsids, may allow for improved expression profiles. Specifically, AAV-5 appears to mediate a more rapid onset of expression²⁴² and may offer a solution to this problem.

One major attraction of a gene transfer approach to the delivery of angiostatic proteins is the possibility of sustained expression following vector delivery. Although Ad vectors mediate relatively short-lived gene expression, Ad-mediated expression of sFlt-1 in the present study was of adequate duration to demonstrate effective expression during retinal angiogenesis. While AAV and lentiviral vectors mediate sustained gene expression in the retina, a sustained therapeutic effect of angiostatic gene transfer is not demonstrable in these models since the neovascularisation is relatively short-lived and for this reason may be investigated only after the development of relevant models of sustained neovascularisation or when applied clinically in human patients.

7.1.2 Tissue patterns of transduction in experimental retinal and choroidal neovascularisation

The vector systems studied exhibit a range of natural tissue tropisms in the eye. For the effective expression of non-secreted proteins the target cell of the expressed protein must itself be transduced. For secreted soluble proteins, in contrast, transduction of a number of cell types might effectively result in the production and secretion of proteins that act

locally or diffuse to act at other intraocular sites. The optimal cell type for local production of a therapeutic protein will be determined by a number of considerations including the efficiency of protein expression, the diffusion characteristics to its site of action and any unwanted effect of transduction or expression on the transduced tissue itself. Despite their contrasting patterns of transgene expression, both Ad- and AAV-mediated expression of sFlt-1 following intravitreal vector delivery resulted in similar reduction in retinal angiogenesis. It is possible that the relative proximity of AAV-transduced cells in the inner retina to areas of retinal neovascularisation may compensate for lower total levels of expression.

The site of vector delivery is also an important consideration. Since AAV-mediated transduction of outer retina tends not to extend beyond the limits of the original area of subretinal injection, the site of retinal transduction can be determined by the controlling the position of the subretinal vector 'bleb'. Laser-induced choroidal neovascularisation at the posterior pole is suppressed following AAV-mediated expression of sFlt-1 in peripheral retina.³⁵⁶ This effect is consistent with diffusion of the secreted angiostatic protein across the retina. Delivery of a similar vector to the posterior retina however, in direct proximity to the site of subsequent choroidal neovascularisation, results in greater angiostatic effect (see Chapter 4). Although closer proximity of angiostatic expression is likely to result in higher local concentrations at the target site, the transduction of more peripheral sites may offer advantages. For example, efficient transduction of the outer retina necessitates the creation of a transient neurosensory retinal detachment with the possibility of damage to photoreceptor cells and attenuation of local retinal function.³⁸²⁻³⁸⁴ Subretinal vector delivery at the retinal periphery may lead to secretion of an angiostatic protein that might diffuse to act effectively at the macula without the need to detach the macula for the sole purpose of vector delivery. In situations where the macula is detached for other reasons, such as macular rotation vector delivery at the site of the neovascular complex itself may be more appropriate.

7.1.3 Efficacy of angiostatic gene transfer in experimental retinal and choroidal neovascularisation

In models of both retinal and choroidal neovascularisation the optimisation of angiogenesis induction and the refinement of techniques for its quantification yielded systems that were sufficiently reproducible to quantify effects of angiostatic gene transfer. Significant angiostatic effects were attainable in both models of ocular angiogenesis by gene transfer of the VEGF-inhibitor sFlt-1. The extent of neovascularisation could be suppressed by up to 50 % compared to untreated control eyes and no evidence of adverse effects was detected in the long term. While the angiostatic effect strongly supports the development of this approach towards clinical application, the question of whether it can be further improved should also be addressed. If the residual neovascular response is the result of incomplete inhibition of VEGF then improved gene delivery systems to increase the efficiency of expression will be valuable. If, on the other hand, the residual response is the result of independent parallel, uninhibited molecular pathways then the combined delivery of appropriate angiostatic proteins in a dual or multiple approach should be considered. Although a VEGF directed gene transfer approach suppresses the progression of evolving neovascularisation in the eye, the possibility that the same approach is able to induce the regression of established new vessels has not yet been investigated. Existing evidence that regression of neovascularisation is associated with down-regulation of VEGF expression suggests that such an effect might be anticipated. While the demonstration of a therapeutic induction of regression effect is difficult in models that are self-limiting, it may be inferred by an acceleration in the rate of spontaneous resolution.³⁵⁹ If the effect on resolution of established new vessels is minimal or slow, then a combined approach involving an ablative step, such as photodynamic therapy or surgical excision, with delivery of angiostatic protein to prevent recurrence in the long term might be an attractive alternative.

7.1.4 Regulation of gene expression in experimental retinal and choroidal neovascularisation

Hypoxia-driven reporter gene expression results in the targeting of sites of active neovascularisation in models of both retinal and choroidal neovascularisation. This system illustrates the potential power of a gene-based delivery system in targeting sites of pathology both spatially and temporally, and presents the possibility of a safeguard against unforeseen adverse effects. Having demonstrated that this system can effectively target expression of a reporter gene, the angiostatic efficiency of HRE-driven expression of sFlt-1 will be determined in the same models in ongoing studies.

7.1.5 Gene transfer in intermediate animal models

Delivery of AAV vector suspensions to the subretinal space of the dog using appropriate microsurgical techniques results in the targeted, efficient and sustained transduction of cells in the outer retina. There is a suggestion, however, of a sustained attenuation of retinal function associated with retinal transduction and GFP reporter gene expression, and it appears that intraocular inflammation (at least in dogs) can develop in association with an immune response to expressed proteins. Investigations have so far concentrated on the transduction of outer retinal cells for the purpose of angiostatic delivery in choroidal neovascularisation. Although we have not yet conducted studies in intermediate animal models to transduce cells of the inner retina for angiostatic delivery in retinal neovascularisation, it seems likely from the observed effects of vector reflux from the subretinal space that this will be feasible by intravitreal vector delivery, with or without vitrectomy.

7.1.6 Rationale for a clinical trial of angiostatic gene transfer

The rationale for a gene transfer approach to retinal angiostasis seems convincing and evidence of its potential value from experimental studies compares favourably with

established and experimental treatments for ocular neovascular disorders. The conventional treatment for ischaemia-induced retinopathy, including proliferative diabetic retinopathy is panretinal laser photocoagulation. This comprises laser ablation of large areas of peripheral retina and though this approach can effectively cause the regression of new vessels it also causes predictable adverse effects on peripheral vision, night vision and contrast sensitivity. Established treatments for choroidal neovascularisation are of only limited efficacy. Laser photocoagulation of choroidal new vessels can effectively delay visual deterioration but is often contra-indicated by foveal involvement. Although photodynamic therapy offers a fovea-sparing alternative in some instances, neither this nor laser photocoagulation offer sustained solutions since their ablative mechanisms do not address the underlying pro-angiogenic stimulus and do not appear to prevent recurrent disease. Newer experimental treatments include repeated intravitreal injections of anti-angiogenic agents including anti-VEGF antibodies⁸¹ and aptamers.⁸⁰ These treatments offer a more rational approach and early results of efficacy in clinical trials are promising. The requirement for repeated intraocular injections to maintain therapeutic levels, however, presents a high cumulative risk of local complications and may present a significant burden on healthcare resources. In the face of the limitations of current treatment options, a gene transfer approach with the potential for a specific and sustained angiostatic effect following a single intraocular procedure is an attractive alternative.

7.2 Development of proposal for clinical trial of angiostatic gene transfer to the eye

The results of studies described in this thesis together with reports from other groups provide substantial evidence that the local gene transfer of angiostatic proteins to the eye can be an effective strategy for the control of angiogenesis in experimental models of both retinal and choroidal neovascularisation. The possibility that such an approach may be similarly effective in patients with ocular neovascular disorders is an exciting prospect. As is the case with any novel therapy, however, the first applications in clinical

trials in humans must be carefully designed to enable an accurate evaluation of clinical efficacy while minimising the possibility of unwanted adverse effects. Careful consideration should be given to selection of the clinical indications for intervention, the appropriate therapeutic protein to be expressed and the optimal choice of vector.

7.2.1 Selection of appropriate target condition for clinical trial of angiostatic gene transfer

The selection of the target condition and indication for intervention is a critical issue. The evaluation of therapeutic effects of novel treatments is most feasible in conditions that have quantifiable features and a relatively predictable natural history. Since any potential risk of the new treatment should be balanced against the consequences of the natural history, conditions with serious consequences and for which current treatment options are relatively ineffective are more appropriately targeted than conditions that are in any way amenable to established treatments. If the safety of new treatments can be first established in a context such as this then the range of indications might be reasonably extended. Neovascular AMD is an attractive target for treatment by local angiostatic gene therapy since it is a common condition that predictably results in deterioration of visual function, has minimally effective current treatments and is anatomically localised such that accurate local delivery of vector is feasible. Quantifiable outcome measures are readily available and these include the extent of neovascularisation as demonstrated by angiography, and visual function as determined by visual acuity, psychophysical techniques and electrophysiology. A proposal for angiostatic gene transfer in diabetic retinopathy, on the other hand is harder to justify at present. Although the current accepted treatment for proliferative diabetic retinopathy is associated with predictable adverse effects, the efficacy of this technique is well established. Only when the possible risks of angiostatic gene transfer are better described will it be ethically acceptable to evaluate the novel therapy in this context.

Although an angiostatic gene transfer approach to neovascular AMD may ultimately offer an effective treatment in isolation of other strategies, its potential may be effectively evaluated as an adjunct to more established techniques. While macular translocation can result in an initial improvement in visual function in patients with subfoveal lesions⁴² recurrent of neovascularisation is an important cause of vision loss.⁴³ The sustained expression, however, of an angiostatic protein following local gene transfer during macular translocation surgery may prevent progression or induce regression of choroidal neovascularisation leading to preservation of vision.

7.2.2 Selection of angiostatic molecule for clinical trial of angiostatic gene transfer

There are a number of promising angiostatic proteins that might be effective in a clinical trial of gene therapy for ocular neovascularisation. The central role of VEGF in ocular neovascular disorders is well characterised and the proven efficacy of anti-VEGF approaches in general, and the soluble VEGF receptor sFlt-1 in particular, make these attractive strategies. Indeed there is already some experience on the efficacy of VEGF-directed approaches in clinical trials including repeated intravitreal injections of an anti-VEGF antibody⁸¹ and of an inhibitory aptamer.⁸⁰ Although the combined angiostatic and neurotrophic properties of PEDF is a particularly attractive combination for application to AMD, the experience of both therapeutic and possible adverse effects of this factor is relatively limited.

7.2.3 Selection of vector for clinical trial of angiostatic gene transfer

The efficacy of AAV vectors for sustained efficient transduction of retinal tissues is well described in many experimental models and these are particularly attractive candidates for gene transfer to the retina. Although the small genome restricts its use to the transfer of smaller genes, AAV vectors efficiently and stably transfect post-mitotic photo-

receptors and RPE cells, are minimally immunogenic and have no known pathogenicity in humans. While production methods in until recently were inefficient, recent improvements in AAV packaging will facilitate the generation of sufficient quantities of vector for clinical trials. Preclinical studies using AAV vectors in the treatment of a variety of inherited monogenic defects and acquired diseases of the retina have been performed. Although AAV vectors have not yet been used in clinical trials of ocular gene transfer, 15 clinical trials (2.4% of all gene therapy trials) are underway for non-ocular conditions. In clinical trials of AAV-mediated gene therapy there has been little or no of evidence of toxicity associated with vector administration or transgene expression and no induction of inhibitory antibodies against the expressed protein.³⁸⁹ Lentiviral vectors present a promising option for future applications²⁶¹ but concerns about the possibility of recombination events leading to the generation of dangerous replication-competent mutants still need to be addressed, and currently AAV vectors are best placed as the vector of choice for clinical trials.

7.2.5 Vector-related toxicity

Having established the efficiency of the vector and the therapeutic effect of the expressed angiostatic protein the critical issues that need to be addressed relate primarily to the safety of the vector and any potential toxicity of the expressed protein. Although for the most part AAV vectors are considered safe, the possibility of insertional mutagenesis and of inadvertent germline transmission cannot be definitively excluded. AAV vectors, unlike the wild-type virus which integrates with high frequency into a single site on human chromosome 19, appear not to integrate site-specifically into the mammalian genome but instead persist as episomes or integrate randomly into the host genome.³⁹⁰⁻³⁹³ The remote possibility of insertional mutagenesis is a concern³⁹⁴ that been heightened recently by the development of lympho-proliferative disorders associated with the use of integrating retroviral vectors in children with subacute combined immunodeficiency. Despite a large number of *in-vivo* studies, however, there is no evidence of insertional mutagenesis by AAV vectors^{395,396} Although the development of hepatocellular carcinoma and angiosarcoma has been described following AAV-mediated gene therapy in mice

with mucopolysaccharidosis VII these tumours are believed to be caused not by an insertional mutagenesis event with subsequent clonal expansion but by overexpression of the gene product.³⁹⁷ The retinal cell types transduced by AAV vectors are non-dividing and proliferative disorders of these tissues are rare; retinoblastoma occurs only in childhood and proliferations of retinal pigment epithelial cells are innocuous. For this reason the likelihood of AAV-mediated retinal tumour development is remote. In our own experience, following subretinal delivery of AAV vectors in many hundreds of rodents, we have found no evidence of tumorigenesis in the eye or elsewhere. Nor have there been any reports following intraocular vector delivery by other groups. In the unlikely event of intraocular tumour development in a clinical trial, frequent monitoring will enable early detection at stage when appropriate prompt management is likely to minimise any associated morbidity.

There has been no evidence of inadvertent germline transmission by AAV vectors despite a number of safety studies in animal models.³⁹⁸ AAV vector sequences have been detected in semen samples following gene delivery of factor IX in patients with haemophilia B, in this study the vector had been administered systemically in relatively large doses and there were no vector sequences detected in the germ cells themselves. The likelihood of inadvertent germline transmission following the microsurgical delivery of tiny volumes of vector to intraocular compartments is likely to be extremely low. The inclusion only of female patients of post-reproductive age in the present study, however, will prevent any consequence of this remote possibility.

The efficacy and toxicity of AAV-mediated retinal gene transfer in intermediate animal models has been the subject of detailed evaluation.^{237,238,278,322,399} Subretinal delivery of AAV-2 vectors in dogs mediates efficient stable reporter gene expression in photoreceptors and retinal pigment epithelial cells. Reporter gene expression in the outer retina is confined to the immediate area of the subretinal delivery and is sustained in cells at this site for at least 18 months. Although there is a suggestion in some cases of an immune response to the expressed reporter gene GFP, immune responses to AAV vector antigens have not been reported. Though a modest attenuation in global retinal function

consistent with toxicity due to high levels of GFP expression has been described in dogs in this thesis, there is no significant sustained effect following localised transient retinal detachment alone, nor following subretinal delivery of AAV vectors in non-human primates,²³⁷ In the proposed clinical trial, short-term induction of a retinal detachment is integral to the surgical procedure of macular translocation and subretinal vector delivery is not expected to significantly delay retinal re-attachment.

7.2.6 Toxicity of the expressed protein

In order to determine the safety of the expressed angiostatic protein a systematic evaluation of biodistribution and toxicology is required. In our initial toxicity studies, following subretinal delivery of AAV.CMV.*sFlt-1* (1×10^{11} particles/ml) in 10 eyes of 5 mice we have detected no evidence of adverse effects up to 12 months as determined by clinical examination, fundus fluorescein angiography or histological evaluation. Once sustained sFlt-1 expression is definitively demonstrated further long-term toxicity studies in larger numbers of rodents will be performed. These will include the evaluation of adverse effects on retinal function, and the measurement of expressed protein in the circulation, that might result either from intraocular expression or expression in non-target organs.

Although further experiments to evaluate the effect of AAV.CMV.*sFlt-1* in large animal models of choroidal neovascularisation may provide additional information such studies may not be essential. The efficacy of similar anti-VEGF approaches has been validated in large animal models of ocular angiogenesis and AAV-mediated reporter gene transfer has been evaluated in dogs and non-human primates. The Gene Therapy Advisory Committee (GTAC) advises that findings from experimental studies in small animals may in some circumstances be extrapolated to minimise the use of larger animals especially non-human primates.

7.2.7 Ethical and regulatory issues

Recent successes in clinical trials of gene therapy⁴⁰⁰ have demonstrated that a thorough and cautious approach to the development of gene-based therapies can lead to effective treatments in patients. However, as the delivery of genes becomes more efficient, the potential for development of significant adverse effects is heightened. Recently, two children effectively cured of X-SCID by retrovirus-mediated gene transfer to bone marrow cells, developed lymphoproliferations that evolved into T cell leukaemia. While the molecular pathogenesis of this process is not yet fully understood, the insertion of the transgene into a recognised T cell proto-oncogene is a clear contributing factor. For vectors that integrate near-randomly into the genome insertional mutagenesis is a finite risk, but only closer molecular scrutiny of patients entering into clinical studies will enable this risk to be quantified. Reassuringly, on the basis of information obtained from multiple animal and human studies performed to date, it would appear that the risk is very low. Clearly the potential for harm has to be carefully balanced against potential for therapeutic benefits, either now or in the future. The GTAC advises that research on human subjects should not put them at disproportionate risk and for this reason should be restricted to patients with serious disorders where current alternative treatments are not wholly effective.⁴⁰¹ Although ocular neovascular disorders are not life threatening, the impact of blindness on quality of life should not be underestimated. Whilst the likelihood of systemic adverse effects following ocular angiostatic gene transfer is low, there is convincing evidence that the potential benefit to vision could be significant.

On the basis of results described in this thesis and work by other groups, a proposal has been developed for a phase I/II clinical trial of AAV-mediated local gene transfer of sFlt-1 in neovascular AMD (Appendix 1: Proposal for phase I/II clinical trial of gene therapy for neovascular AMD). The proposed clinical trial is an open-label, dose-escalation study to investigate the safety, tolerability and potential activity of AAV-mediated local expression of sFlt-1 following delivery of the vector suspension to the subretinal space during macular translocation surgery.

7.3 Future directions

Having designed a clinical trial of gene therapy for neovascular age-related macular degeneration based on the current evidence from experimental and pre-clinical studies, future developments in a number of specific areas would facilitate improvements in the efficacy and safety of this approach.

7.3.1 Experimental models of retinal and choroidal neovascularisation

Although angiostatic gene transfer offers the potential of sustained long-term suppression of angiogenesis, this possibility is currently difficult to confirm experimentally for lack of appropriate models. The majority of models of ocular neovascularisation currently available comprise short-lived angiogenic responses. The development of longer-term models of retinal and choroidal neovascular disorders that more closely reflect clinical conditions would offer an ideal context in which to refine a gene therapy strategy.

Rodent models of diabetes and sickle cell disease may be useful for the evaluation of angiostatic therapies though the neovascularisation in these animals tends to develop over a very prolonged timecourse and is of limited reproducibility. A spontaneously diabetic strain of the Sprague-Dawley rat has been recently described that develops tractional retinal detachment with fibrous proliferation but these changes occur up to 70 weeks of age.³¹¹ Retinal neovascularisation occurs in a transgenic mouse model of sickle cell disease but only in 30 % in animals older than 15 months of age.⁴⁰²

Sustained delivery of angiogenic factors can be achieved by the introduction of slow release devices⁴⁰³ or by overexpression using vectors or transgenic animals. Adenovirus-mediated overexpression of VEGF in retinal pigment cells in the rat can induce choroidal neovascularisation that extends through defects in Bruch's membrane into the subretinal space⁷² but this model is insufficiently reproducible for the evaluation of potential

angiostatic therapies. Sustained overexpression of VEGF in photoreceptor cells in transgenic mice induces neovascularisation that originates from the deep capillary bed of the retina and extends beneath the photoreceptor layer into the subretinal space to form clumps of blood vessels surrounded by proliferated retinal pigmented epithelial cells^{71,309} whereas, in contrast, VEGF overexpression in the retinal pigment epithelium induces intrachoroidal neovascularisation.³³² In neither case, however, does the neovascularisation penetrate an intact Bruch's membrane and it seems that additional insults to the integrity of Bruch's membrane are required to induce growth of choroidal vessels into the subretinal space as seen in age-related macular degeneration. Laser rupture of Bruch's membrane may promote neovascular penetration in this context and offer a more relevant sustained model of choroidal neovascularisation in mice.⁴⁰⁴

Although many models of choroidal neovascularisation induced by delivery of growth factors or rupture of Bruch's membrane have been described, there are few that reproduce the changes in the extracellular matrix characteristic of AMD. One example is APO(*)E3- Leiden mice that spontaneously develop basal laminar deposit, a phenotype that is enhanced by a high fat content diet.⁴⁰⁵ Dissappointingly, however, these animals fail to develop either drusen or choroidal neovascularisation.

Although large animals provide a valuable context in which to evaluate potential toxicity of novel therapies, to date there is minimal experience of therapeutic gene transfer in large animal models and more relevant models of retinal or choroidal neovascularisation in intermediate animals are required. Established models include laser-induced choroidal neovascularisation³³⁶ and laser retinal vein occlusion-induced iris neovascularisation⁶⁴ in primates, and retinal neovascularisation following laser retinal vein occlusion in miniature pigs^{312,313} Although existing models are valuable, neovascularisation in each case occurs in response to a single insult and is relatively short-lived. Sustained delivery of VEGF in primates that might be expected to induce a longer term response fails to induce neovascularisation in the retina.³¹⁶ A primate model for age related macular drusen has been described but these animals do not develop choroidal neovascularisation. Since an ideal pre-clinical model of sustained retinal or choroidal neovascularisation is still

lacking, any therapeutic effect of novel angiostatic agents in the long-term may become evident only in clinical trials in patients.

7.3.2 Novel strategies for gene-based therapies

Emerging alternatives to cDNA-based strategies for gene transfer may offer improved specificity or efficacy. One example that is currently the subject of ongoing studies is zinc finger protein (ZFP) transcription factors. These factors are designed *de novo* to bind to specific targeted DNA sequences and offer a novel gene therapy approach to achieve the activation or repression of endogenous genes.⁴⁰⁶ This approach facilitates the simultaneous regulation of multiple separate genes with a single therapeutic intervention, either by designing a ZFP that binds a DNA sequence common to all the desired genes or by combining expression cassettes encoding multiple ZFP constructs into a single gene-delivery vehicle. The relatively small size of the ZFP cassettes (0.6-1.0kb) facilitates the latter approach. The effect of upregulation of endogenous PEDF expression using lentivirus-mediated expression of ZFPs on experimental choroidal neovascularisation is currently being evaluated.

Another novel approach is RNA interference (RNAi), a biological strategy that results in specific silencing of targeted genes through expression of small interfering RNA. This can lead to markedly diminishing of expression of exogenous and endogenous genes in vitro and in vivo. A viral-mediated RNAi strategy may prove generally useful in reducing expression of target genes to provide therapy for dominant human diseases.⁴⁰⁷ If the transduction efficiency of target cells can be optimised this strategy may be applied to directly down-regulate the expression of endogenous angiogenic growth factors in contrast to more conventional techniques that aim to over-express proteins that inhibit angiogenesis. In practice, however, a significant effect might be difficult to achieve, as transduction of a substantial proportion of target cells is likely to be required.

7.3.3 Improved vectors for angiostatic gene transfer

The selection of an ideal vector will depend on the clinical condition to which it is applied. For many ocular neovascular disorders the ideal vector will be tissue-targeted and will efficiently mediate expression that is of rapid onset and sustained duration without inducing significant immune responses. Although AAV vectors fulfil many of these criteria, maximal expression by AAV-2 vectors is not reached for several weeks. Pseudotyping by AAV-5 offers the possibility of vectors that mediate significant expression within a few days.²⁴²

7.3.4 Improved tissue-targeting and regulation of angiostatic gene expression

Although the ideal angiostatic agent will be both powerfully effective and non-toxic this combination may be difficult to achieve in practice. Long-term safety will be optimised by the development of effective systems for tissue-targeting and regulated-expression. A number of strategies to restrict gene expression to endothelial cells have been described. The use of retroviral vectors to specifically transduce proliferating endothelial cells at sites of laser-induced choroidal neovascularisation³⁵³ has been discussed previously. Alternatively, expression can be restricted specifically to transduced vascular endothelial cells through the use of endothelium-specific promoters such as the KDR⁴⁰⁸ or von-Willebrand factor promoters.³⁰¹ A novel strategy for angiogenesis-targeted gene therapy is the generation of angiostatin from endogenous plasminogen by protease gene transfer.⁴⁰⁹ Since angiogenesis is associated with overexpression of matrix metalloproteinases (MMPs), protease-activated systems such as this can result in selective gene delivery to sites of active neovascularisation.³⁰⁶

The incorporation of regulatable elements, whether pharmacological or tissue-responsive, into the vector construct will add additional safeguards against the possibility of adverse effects due to inappropriate transgene expression. The results of experiments described in this thesis demonstrate that expression can be targeted spatially and temporally to sites of

angiogenesis but regulation of the absolute level of expression would further refine the approach. Dose regulation may be facilitated by pharmacologically-induced systems. The relatively poor penetration of doxycycline across the blood retinal barrier may be overcome by focal breakdown in the context of choroidal neovascularisation (or in macular oedema) such that expression at those sites might be induced effectively, and possibly, in proportion to the degree of drug penetration.

The hypoxia response pathway offers attractive opportunities to target angiogenesis. Gene transfer of antisense hypoxia inducible factor-1 alpha is one approach that has been effective against tumour angiogenesis.²⁹³ Gene targeting to sites of active angiogenesis in laser-induced choroidal neovascularisation can be achieved by HRE-driven expression.³⁶² The role of hypoxia in AMD, however, is not well described and this particular system may not necessarily be effective in this context. The identification, however, of alternative features in choroidal neovascularisation might enable the regulation of gene expression in response to the tissue environment.

7.3.5 Future directions for angiostasis in diabetic retinopathy and AMD

In diabetes the pathways by which hyperglycaemia leads to retinal neovascularisation offer further opportunities for therapeutic intervention. Local gene transfer of proteins targeted to aldose reductase, AGEs, or PKC is theoretically possible and gene targeting of these upstream events offers a potentially powerful means to prevent or arrest proliferative diabetic retinopathy.

Additional strategies may address ischaemia-induced neovascularisation by preventing retinal hypoxia or even by inducing appropriate revascularisation of ischaemic retina. Gene targeting of NFkB may help to prevent retinal hypoxia through the inhibition of vascular cell apoptosis. A highly attractive approach to ischaemia-induced retinal neovascularisation would be to induce appropriate revascularisation of the ischaemic retina. If this can be achieved, not only might it prevent or arrest sight-threatening pre-

retinal neovascularisation through down-regulation of VEGF expression, but it may also improve function of the ischaemic tissue. Nitric oxide has been implicated in the control of physiological and pathological angiogenesis.¹⁷⁵ Evidence suggests that inducible nitric oxide synthase (iNOS) is responsible for mediating the change from retinal to pre-retinal neovascularisation in ischaemic retinopathy.¹⁷⁹ Gene transfer of an inhibitor of iNOS would be a highly attractive strategy that might control vitreal neovascularisation through improvement in the vascularisation of the hypoxic retina.

Local gene transfer of sFlt-1 may have valuable applications beyond neovascularisation in diabetic retinopathy and age-related macular degeneration. In addition to its central role in angiogenesis,⁶¹ VEGF also promotes vascular permeability through Flt-1 receptors.⁴¹⁰ By reducing vasopermeability, VEGF antagonists such as sFlt-1 may have additional potential applications in the management of macular oedema in diabetes,⁴¹¹ in uveitis⁴¹² and after cataract surgery.⁴¹³

In age-related macular degeneration, changes in the extracellular matrix may offer opportunities for therapeutic intervention. Although the role of matrix metalloproteinases in angiogenesis is complex,¹²⁷ local gene transfer of an appropriate TIMP, for example,³⁵⁵ might protect against the matrix dissolution that is a critical feature of this process. The concept that oxidative stress has an important role in the pathogenesis of AMD through RPE cell dysfunction, accumulation of lipofuscin, and the modulation of angiogenic factors (reviewed by Beatty *et al.*⁴¹⁴) suggests the possibility of additional upstream targets, though many anti-oxidants may perhaps be effectively and safely administered systemically without a requirement for local delivery. A evolving body of evidence suggests that subclinical inflammation may also play an important role in the development of neovascular AMD; leucocyte adhesion and macrophage activation may be legitimate targets for therapeutic intervention, for example by local inhibition of intercellular adhesion molecule-1 (ICAM-1) or monocyte chemoattractant protein-1 (MCP-1).

Loss of visual function in AMD is not only the result of choroidal neovascularisation but is also caused by atrophy of retinal pigment epithelial cells. Local gene transfer of neurotrophic factors offers one potential strategy that might slow cell loss. PEDF is a particularly attractive candidate for this role as it displays both angiostatic and neuroprotective properties.^{360,415} Since, however, patients with AMD often present with established retinal pigment epithelial cell atrophy, cell-replacement strategies may be required in order to restore visual function. Transplants of retinal pigment epithelial cells or stem cells are potential approaches designed to address this problem. If the challenges of graft rejection or cell differentiation can be successfully met, then such strategies may be combined with the *ex vivo* transduction of transplanted cells such that the sustained expression of neuroprotective or angiostatic factors might improve the long-term prognosis.

While these strategies offer avenues for future research, the current understanding of the biology of angiogenesis has already facilitated the building of a substantial body of evidence supporting the hypothesis that local gene delivery of rationally selected proteins can result in a reduction in neovascularisation of the retina and choroid. The immediate challenge is to extend this approach from experimental models in the laboratory to patients in the clinic. The translation of this novel, potentially powerful, gene-based therapy from pre-clinical research to clinical trials presents considerable ethical and logistical difficulties that should not be underestimated. It is hoped, however, that clinical application of this approach in the near future will prove safe and effective.

Appendix

Proposal for a phase-1 clinical trial of gene therapy for neovascular age-related macular degeneration

An open-label phase 1 single-administration study of subretinal AAV.CMV.sFlt-1 in neovascular age-related macular degeneration.

A: Objectives and rationale

Age-related macular degeneration (AMD) is the commonest cause of legal blindness in industrialized countries, with an estimated incidence of 20 000 new cases annually in the UK and prevalence of 1.9% in people older than 50 years.¹² The pathogenesis of AMD involves abnormalities of the extracellular matrix at the level of Bruch's basement membrane.¹³ Eighty percent of patients with severe visual loss due to AMD have the neovascular form of the condition^{19,20} in which pathological neovascularisation under the retina causes loss of vision through exudation or haemorrhage that disrupts the normal interaction between photoreceptor cells and retinal pigment epithelial cells.

The established treatment modalities for choroidal neovascularisation in AMD comprise thermal laser photocoagulation and photodynamic therapy. These ablative approaches can offer short-term benefit to certain subgroups of patients²¹⁻²³ but are associated with significant adverse effects.^{10,24} Laser photocoagulation can be beneficial for the small minority of patients who have well-demarcated ("classic") neovascular complexes that do not extend beneath the fovea^{24,25} but is not helpful for the majority of lesions that involve the fovea since the associated destruction of foveal photoreceptor cells predictably results in an immediate and irreversible loss of central vision.²⁴ Photodynamic therapy is a non-thermal process that aims to selectively destroy new blood vessels by irradiation

following the intravascular administration of a photosensitising agent. Since this approach is relatively selective for vasculature and tends not to result in the destruction of overlying photoreceptor cells, it can be effective in patients with subfoveal neovascularisation and delays loss of vision in a subgroup of patients with predominantly classic subfoveal lesions. Both photocoagulation and photodynamic therapy are designed to eliminate abnormal blood vessels, but since these treatments fail to address the underlying stimuli for blood vessel growth they are associated with high rates of persistent and recurrent disease. Following photocoagulation the cumulative proportion of treated eyes with recurrent/persistent choroidal neovascularisation is approximately 50% after 3 years²⁶ and is accompanied by an increased frequency of severe visual loss.²⁷ Persistence and recurrence neovascularisation are also common after photodynamic therapy, requiring frequent further treatments.²² Experimental surgical procedures are being developed to relocate the neurosensory retina overlying a foveal neovascular lesion outside the border of the lesion in order to maintain or recover its function.⁴¹⁶ Moving the fovea away from a subfoveal choroidal neovascular lesion also permits the removal of the lesion or its destruction by laser photocoagulation with minimal risk to the fovea.⁴² Recurrence of neovascularisation following macular translocation, however, is common and is an important cause of vision loss.⁴³

The limitations of available treatments for ocular neovascular disorders underlie the clinical need to develop rationally designed novel approaches that are directed against the underlying pro-angiogenic stimuli so as to achieve a sustained therapeutic effect. The development of such treatments depends on a clear understanding of the molecular and cellular processes involved in angiogenesis. Angiogenesis is a complex multi-step process that involves the out-sprouting of vascular endothelial cells from existing vessels through endothelial cell proliferation, extracellular matrix remodelling, endothelial cell migration and capillary tube formation. This process is controlled by complex interactions between growth factors, extracellular matrix and cellular components, the net outcome being determined by the balance of angiogenic and angiostatic elements.⁵⁰ Molecules that have attracted particular interest as potential targets for angiostatic approaches in ocular disease include the pro-angiogenic cytokine vascular endothelial

growth factor and the anti-angiogenic pigment epithelium-derived factor. The therapeutic manipulation of one or a combination of these molecules offers the potential means to control neovascularisation in the eye.

Vascular endothelial growth factor (VEGF) is a potent endothelial cell-specific mitogen that plays a critical role in angiogenesis.^{51,52} VEGF is a 46kDA homodimeric glycopeptide that is expressed by several different ocular cell types including pigment epithelial cells, pericytes, vascular endothelial cells, neuroglia and ganglion cells,^{18,51,53,54} and in specific spatial and temporal patterns during retinal development.⁵⁵ Expression of VEGF is upregulated by hypoxia *in vitro*⁵⁶ and *in vivo*.⁵⁴ It acts via specific *fms*-like receptors, *Flt-1* and *Flk-1* /KDR, which are high-affinity receptor tyrosine kinases expressed on vascular endothelial cells and leads to endothelial cell proliferation, migration, and increased vasopermeability. VEGF levels are increased in experimental models of retinal ischaemia,^{53,64} in patients with proliferative diabetic retinopathy,⁶⁵⁻⁶⁷ retinopathy of prematurity⁶⁸ and retinal vein occlusion.⁶⁶ A soluble truncated form of the VEGF receptor *Flt-1*, *sFlt-1*, is the only known endogenous specific inhibitor of VEGF^{76,77} The angiostatic activity of *sFlt-1* results from inhibition of VEGF by two mechanisms, causing sequestration of VEGF and forming inactive heterodimers with the membrane-spanning isoforms of the VEGF receptors *Flt-1* and *KDR*.^{59,61} *sFlt-1* has attracted particular attention for its potential therapeutic role in the control of neovascularisation.^{59,60,76,417} It is clear that angiogenesis is a highly complex process and that the effects of cytokines on endothelial cell-matrix interactions can be contextual.¹⁹⁵ Since the determination of the angiogenic phenotype, however, appears to be the result of a net balance of positive and negative regulators of blood vessel growth¹⁹⁶ the introduction of a single agent to tip the balance towards angiostasis may be all that is required to achieve a therapeutic effect.

Though potentially efficacious, the systemic administration of angiostatic proteins risks important adverse systemic effects. In addition to its critical role in embryogenesis, physiological angiogenesis is central to wound healing and recovery from ischaemic events through revascularisation and the formation of collateral circulations. Patients with

retinal neovascular disease, typically associated with diabetes or advanced age, are also likely to be at increased risk of ischaemic heart disease, cerebrovascular and peripheral vascular disease. The systemic inhibition of angiogenesis in these individuals risks compromising critical vascular responses to ischaemic events. In addition to this, the production of recombinant proteins is technically difficult and their manufacture is expensive.¹⁶⁵ Since access of proteins to the retina is restricted by the blood-retinal barrier, the high systemic doses required to achieve therapeutic intraocular levels would be particularly expensive and hazardous.

For these reasons, the local delivery of angiostatic agents offers significant potential advantages. Intraocular neovascularisation in experimental models is reduced by the repeated intravitreal injections of neutralizing anti-VEGF monoclonal antibodies,⁷³ recombinant soluble VEGF-receptor chimeric proteins⁶⁰ and antisense oligodeoxynucleotides.⁷⁴ The effective control of angiogenesis in patients with retinal neovascular disorders, however, is likely to require the sustained presence of the angiostatic protein in the eye. The relatively short half-life of proteins delivered by intravitreal injection is such that frequently repeated administration would be necessary to maintain therapeutic levels¹⁹⁹ and would pose a high cumulative risk of local complications including intraocular infection, vitreous haemorrhage and retinal detachment. In contrast, somatic gene transfer offers the possibility of localised, targeted, sustained and regulatable delivery of therapeutic proteins following a single procedure to introduce a vector to an appropriate intraocular site. Using a gene transfer approach, sustained expression can be achieved locally while minimising any risk of systemic adverse effects. Tissues may be targeted by vector design and surgical techniques, and expression may be regulated through the use of tissue-specific, inducible or tissue-responsive promoters.

The eye is an ideal organ for *in-vivo* gene transfer. Ocular anatomy lends itself to the accurate delivery of vector suspensions because the tissues are compartmentalised and readily accessible by microsurgical techniques under direct visualisation. Its small size means that only tiny volumes of vector suspensions are required to transduce a significant

proportion of cells in the target tissue, and even non-dividing cell populations may be efficiently transduced by a single dose. The relative immune privilege of many ocular tissues may confer an advantage in terms of long-term transgene expression.⁴¹⁸ The optical transparency of the eye enables GFP reporter gene expression to be observed *in-vivo* in many instances, and therapeutic effects on structure and function may be readily observed, recorded and quantified using a variety of techniques both experimentally and clinically.

A number of different viral and non-viral vector systems for gene transfer to ocular tissues have been extensively evaluated.^{201,202} Recombinant adeno-associated virus (AAV) vectors are particularly attractive for their lack of significant pathogenicity and the potential for long-term transduction of cells in the outer retina.^{235-237,243} and the means to produce high-titer vectors for clinical application have been developed.²²⁸ AAV-mediated local expression of *sFlt-1* by somatic gene transfer offers the possibility of localised, targeted, and sustained delivery to the retina following a single procedure to introduce a vector suspension to an appropriate intraocular site. This approach is effective in experimental models of retinal³⁶² and choroidal neovascularisation³⁵⁶ and we have shown that AAV-mediated gene transfer leads to efficient sustained expression in an intermediate animal model model.³⁹⁹

Neovascular AMD is an attractive target for treatment by local angiostatic gene therapy since it is a common condition that predictably results in deterioration of visual function, has minimally effective current treatments and is anatomically localised such that accurate local delivery of vector is feasible. Although an angiostatic gene transfer approach may ultimately offer an effective treatment in isolation of other strategies, its potential may be effectively evaluated as an adjunct to more established techniques. While macular translocation can result in an initial improvement in visual function in patients with subfoveal lesions⁴² recurrent of neovascularisation is an important cause of vision loss.⁴³ Our hypothesis is that the sustained expression of an angiostatic protein following local gene transfer during macular translocation surgery may prevent progression or induce regression of choroidal neovascularisation leading to preservation

of vision. The proposed clinical trial is an open-label, dose-escalation phase 1 study to investigate the safety, tolerability and potential activity of AAV-mediated local expression of sFlt-1 following delivery of the vector suspension to the subretinal space during macular translocation surgery.

B: Patient population

An important ethical consideration relevant to this study is the proposal of a powerful novel technology in a condition that is not life threatening. Gene therapy research on human subjects should not put them at disproportionate risk and for this reason should be restricted to patients with serious disorders where current alternative treatments are not wholly effective. Although age-related macular degeneration is not life threatening, current treatments are of very limited efficacy and the impact of visual disability on quality of life should not be underestimated. Whilst pre-clinical data suggest that the likelihood of systemic adverse effects following ocular angiostatic gene transfer is low, experimental data suggests this approach may offer a significant benefit to visual function.

The possibility that local expression of the angiostatic protein might result in adverse effects on visual function in the human eye is minimised by the inclusion only of subjects in whom the visual prognosis is otherwise poor. Whilst any risk of inadvertent germline transmission following intraocular administration of a small dose of vector suspension is believed to be extremely low, this remote possibility will be prevented in this study by the inclusion only of postmenopausal women.

The additional procedures that this trial requires over and above normal clinical care relate to the delivery of the vector suspension and the subsequent monitoring for therapeutic and potential adverse effects. The subretinal delivery of vector is a relatively uncomplicated procedure. This is unlikely to extend the duration of the surgical procedure by more than 20 minutes and is not likely to introduce significant additional surgical risk. Monitoring of patients following the delivery of viral vector will involve

frequent attendance for examination in the outpatients department and may require additional investigations such as venepuncture and fundus fluorescein angiography.

C: Gene construct and delivery system

The DNA construct proposed for delivery in this trial is AAV.CMV.*sFlt-1*. AAV vectors are well established as the vector of choice for efficient transduction of photoreceptor cells and mediate sustained gene expression in the long term.²³⁵⁻²³⁷ These vectors efficiently and stably transduce post-mitotic photoreceptors and RPE cells, are minimally immunogenic and have no known pathogenicity in humans. Preclinical studies using AAV vectors in the treatment of a variety of inherited monogenic defects and acquired diseases of the retina have been performed. In clinical trials of AAV-mediated gene therapy there has been little or no evidence of toxicity associated with vector administration or transgene expression and no induction of inhibitory antibodies against the expressed protein.³⁸⁹

The vector backbone for this study is on recombinant adeno-associated virus AAV type -2 (AAV-2). This consists of the inverse terminal repeats (ITRs) flanking the therapeutic transgene and its promoter. The therapeutic transgene encodes the soluble truncated VEGF receptor *sFlt-1*. A cytomegalovirus (CMV) promoter facilitates gene expression in both retinal pigment epithelial cells and photoreceptor cells following subretinal vector delivery. The construct map and complete nucleotide sequence are described in the appendices (in preparation). The vector will be delivered in the form of a suspension of viral particles injected directly into the subretinal space using a 34-gauge cannula. This will be performed during intraocular surgery under direct observation using an operating microscope.

Although for the most part AAV vectors are considered safe, the possibility of insertional mutagenesis and of inadvertent germline transmission cannot be definitively excluded. AAV vectors, unlike the wild-type virus which integrates with high frequency into a single site on human chromosome 19, appear not to integrate site-specifically into the

mammalian genome but instead persist as episomes or integrate randomly into the host genome.³⁹⁰⁻³⁹³ The remote possibility of insertional mutagenesis is a concern³⁹⁴ that been heightened recently by the development of lympho-proliferative disorders associated with the use of integrating retroviral vectors in children with subacute combined immunodeficiency. Despite a large number of in-vivo studies, however, there is no evidence of insertional mutagenesis by AAV vectors^{395,396} Although the development of hepatocellular carcinoma and angiosarcoma has been described following AAV-mediated gene therapy in mice with mucopolysaccharidosis VII these tumours are believed to be caused not by an insertional mutagenesis event and subsequent clonal expansion but by overexpression of the gene product.³⁹⁷ The retinal cell types transduced by AAV vectors are non-dividing and proliferative disorders of these tissues are rare; retinoblastoma occurs only in childhood and proliferations of retinal pigment epithelial cells are innocuous. For this reason the likelihood of AAV-mediated retinal tumour development is remote. In our own experience, following subretinal delivery of AAV vectors in many hundreds of rodents, we have found no evidence of tumorigenesis in the eye or elsewhere. Nor have there been any reports following intraocular vector delivery by other groups. In the unlikely event of intraocular tumour development in a clinical trial, frequent monitoring will enable early detection at stage when appropriate prompt management is likely to minimise any associated morbidity.

There has been no evidence of inadvertent germline transmission by AAV vectors despite a number of safety studies in animal models.³⁹⁸ AAV vector sequences have been detected in semen samples following gene delivery of factor IX in patients with haemophilia B, in this study the vector had been administered systemically in relatively large doses and there were no vector sequences detected in the germ cells themselves. The likelihood of inadvertent germline transmission following the microsurgical delivery of tiny volumes of vector to intraocular compartments is likely to be extremely low. The inclusion only of postmenopausal women in the present study, however, will prevent any effect of this remote possibility.

D: Prior studies

Though there is currently no animal model of age-related macular degeneration, choroidal neovascularisation can be induced by laser-induced rupture of Bruch's basement membrane³³⁶ or the local delivery of pro-angiogenic cytokines including VEGF^{71,332} and bFGF.¹¹⁴ Laser-rupture of Bruch's membrane results in the development of a neovascular complex that extends from the choroidal vasculature through the membrane into the subretinal space and the extent of this response can be quantified by *in-vivo* fluorescein angiography. A number of different viral and non-viral vector systems for gene transfer to ocular tissues have been extensively evaluated^{201,202} but AAV vectors are particularly attractive vectors for gene transfer in the treatment of choroidal neovascularisation since they lack significant pathogenicity and have the potential to efficiently and stably transduce photoreceptors and retinal pigment epithelial cells which are both in close proximity to the developing neovascular complex.^{235-237,243} AAV-mediated local expression of *sFlt-1* by somatic gene transfer offers the possibility of localised, targeted, and sustained delivery to the retina following a single procedure to introduce a vector suspension to an appropriate intraocular site. Having previously demonstrated that intraocular injection of AAV.CMV.*sFlt-1* results in the reduction of angiogenesis in experimental ischaemia-induced retinal neovascularisation³⁶² we have also found that delivery of the same vector results in a reduction in choroidal neovascularisation by up to 40% (unpublished data). The therapeutic efficacy of this approach has been confirmed by other groups using both AAV vectors³⁵⁶ and adenovirus vectors.⁷⁵ AAV vectors have also been used to effectively suppress choroidal neovascularisation by expression of alternative anti-angiogenic factors including PEDF^{359,360} and angiostatin.³⁰² In toxicity studies, following subretinal delivery of AAV.CMV.*sFlt-1* (1x10¹¹ particles/ml) in 10 eyes of 5 mice we have found no evidence of adverse effects up to 12 months as determined by clinical examination, fundus fluorescein angiography or histological evaluation.

AAV-mediated retinal gene transfer has also been evaluated in intermediate animal models.^{237,238,278,322} Our own studies have demonstrated that subretinal delivery of AAV-2

vectors in dogs mediates efficient stable reporter gene expression in photoreceptors and retinal pigment epithelial cells. Reporter gene expression in the outer retina is confined to the immediate area of the subretinal delivery and is sustained in cells at this site for at least 18 months.³⁹⁹

E: Study protocol

Study design:

A non-randomised, dose-escalation, single administration study of subretinal AAV.CMV.*sFlt-1* during macular translocation surgery for neovascular age-related macular degeneration.

Inclusion criteria:

Patients undergoing macular translocation for neovascular age-related macular degeneration will be invited to participate in the study.

Exclusion criteria:

Male patients or pre-menopausal female patients.

Co-existing ocular disease.

Recruitment procedure:

Individuals eligible for inclusion will be recruited from the Accident and Emergency and out-patients departments at Moorfields Eye Hospital, London and St. Paul's Eye Unit, Liverpool. It is anticipated that approximately 2 patients per month will be enrolled and total of 12 patients overall will be recruited to the study.

Consent:

Independent counselling will be offered prior to informed signed consent and study enrolment (see patient information and consent forms –in preparation).

Administration procedure:

Following macular translocation, vector suspension (AAV.CMV.*sFlt-1*) will be administered under the neurosensory retina at the site of the choroidal neovascular membrane. After a period of 15 minutes, residual vector suspension will be aspirated and the retinal reattachment maintained by silicone oil tamponade.

Dose-escalation:

The starting dose will be 1×10^9 viral particles per ml (total of 2.5×10^8 particles units) (2 log units lower than the dose safe predicted by pre-clinical studies) and the dose will be increased to 1×10^{10} and 1×10^{11} particles per ml. Four patients will be treated at each dose level. Dose escalation will take place only after the safety and tolerability at the lower dose has been carefully evaluated by clinical assessment of visual function and intraocular inflammation and retinal angiography. No further patients will be dosed following an unexpected clinically meaningful grade IV toxicity until monitoring of safety data have been adequately reviewed.

Post-operative management:

It is anticipated that patients will be discharged from hospital and allowed home within a few days of the surgical procedure. Patients will be prescribed conventional postoperative medication comprising topical antibiotic, anti-inflammatory and mydriatic medication. Monitoring of patients will be undertaken in the out patients departments at Moorfields Eye Hospital or at St Paul's Eye Unit, initially on a weekly basis and subsequently at longer intervals.

Methods to determine whether gene sequences are inserted and expressed:

The retina is relatively inaccessible to sampling or biopsy, and sampling of retinal tissue at the transduced macula would result in a predictable adverse effect on visual function. For these reasons sequence insertion and gene expression in ocular tissues will not routinely be determined.

Safety parameters:

Complications of intraocular surgery will be detected by routine postoperative examination and managed according to conventional techniques. The possibility of immune responses leading to intraocular inflammation will be carefully considered. Such responses will be anticipated through the use of prophylactic topical steroids. The development of progressive intraocular inflammation will be addressed by treatment with intensive topical steroids and if necessary systemic corticosteroid medication.

The extent of choroidal neovascularisation will be determined by clinical examination and fundus fluorescein angiography. Any progression despite subretinal administration of AAV.CMV.sFlt-1 will be treated promptly by direct argon laser photocoagulation before central vision is threatened by subfoveal extension.

The possibility of intraocular inflammation due to immune responses to the vector or expressed protein will be minimised by standard postoperative prophylactic treatment using topical corticosteroids. Any progressive intraocular inflammation will be addressed by treatment with intensive topical steroids and if necessary systemic corticosteroid medication. The possibility that local expression of the angiostatic protein might result in adverse effects on visual function in the human eye is minimised by the inclusion only of subjects in whom the visual prognosis is otherwise poor.

Serum sample will be taken at defined intervals for determination of serological responses and possible presence of viral particles in the circulation. The possibility of inadvertent insertion of vector sequences in non-target cells will be investigated by PCR on DNA of circulating leukocytes. Whilst any risk of inadvertent germline transmission following intraocular administration of a small dose of vector suspension is believed to be extremely low, the remote possibility will be prevented in this study by the inclusion only of females of post-reproductive age.

Public health considerations:

Exposure of health workers to the vector suspension will be minimised by the implementation of appropriate measures in the operating theatres. The vector will be maintained in a sealed container until required by the surgeon for intraocular injection. Following vector delivery into the eye the operating field will be irrigated using saline solution in order to mitigate the possible presence of vector particles refluxed from the eye. The vector used does not contain replication competent viruses and the potential for transmission of vector sequences from the patient to other persons is extremely small.

Efficacy parameters:

The primary outcome measure for this study will be progression of choroidal neovascularisation at the site of vector administration. The extent of the neovascular complex will be determined by clinical examination and fundus fluorescein angiography. A secondary outcome measure will be visual function as determined by visual acuity.

Statistical analysis:

As this is a Phase I dose escalation study, no formal analysis will be employed. Results will be tabulated but statistically valid comparisons will not be attempted.

Anticipated total number of patients and sites:

Up to a total of 12 patients will be recruited between the 2 investigational sites.

Anticipated start and completion dates: 2004/2005

F: Nature of the research sites:

The ocular gene therapy group at the Institute of Ophthalmology has a longstanding collaboration with Professor Adrian Thrasher at the Institute of Child Health, UCL and the combined groups have valuable experience of clinical trials of gene therapy and monitoring of patients following treatment using viral vectors. The Institute of Ophthalmology is one of the leading centres for vision research internationally and was awarded a 5* rating in the most recent research assessment exercises. Moorfields Eye

Appendix

Hospital itself awarded three-star Trust status by the Department of Health, has an established reputation for outstanding clinical research and together in partnership with the Institute of Ophthalmology manages the largest ongoing ophthalmic research programme in the world. This collaboration, based on a single site, provides a unique opportunity for the development of effective treatments of eye disease. The hospital is fully equipped for the clinical evaluation and investigation of patients with age-related macular degeneration and for vitreoretinal surgery for the intraocular delivery of vector suspensions. Essential support services on site include an electrodiagnostic facility, clinical photography and pathology. Immunology and microbiology services are provided locally at Guy's and St.Thomas' Hospital.

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