Gene therapy for ocular angiogenesis

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ABSTRACT

Ocular neovascularization is a central feature of diabetic retinopathy and age-related macular degeneration. These conditions are the major causes of blindness in the developed world. Current treatments are of limited efficacy and associated with significant adverse effects. Characterization 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 the effect of their intraocular injection is short-lived. Local gene transfer, however, offers the possibility of targeted, sustained and regulatable delivery of angiostatic proteins to the retina after a single procedure to introduce a vector to an intraocular site. The effect of intra-ocular delivery of recombinant viruses carrying genes encoding angiostatic proteins has been demonstrated in rodent models of ocular neovascularization. Recombinant adeno-associated virus-mediated local gene transfer of a vascular endothelial growth factor inhibitor controls both retinal and choroidal neovascularization. The clinical application of this approach may require the means to regulate gene expression in order to minimize the potential for adverse effects. Regulation of transgene expression by means of a hypoxia-responsive promoter offers an attractive strategy for the targeted and regulated delivery of angiostatic proteins to the retina in the management of ischaemia-induced ocular neovascularization. Preclinical studies of gene transfer in a large animal model following subretinal delivery of a recombinant adeno-associated virus vector have demonstrated efficient sustained reporter gene expression in cells of the outer retina. Recent progress has enabled the planning of clinical trials of gene therapy for ocular neovascular disorders.

INTRODUCTION

Angiogenesis is a complex multi-step process that involves 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 revascularization of ischaemic tissues. Angiogenesis is also a central feature of many important diseases, including cancer, rheumatoid arthritis, atherosclerosis and ocular neovascularization. Pathological angiogenesis occurs in retinopathy of prematurity, proliferative diabetic retinopathy and age-related macular degeneration; the leading causes of blindness in infants, individuals of working age and the

Key words: angiogenesis, gene therapy, neovascularization, recombinant adeno-associated virus, retina.

Abbreviations: Ad, adenoviral; AGE, advanced glycation end-product; AMD, age-related macular degeneration; Flt, fms-like tyrosine kinase; GH, growth hormone; HRE, hypoxia-response element; IGF-1, insulin-like growth factor-1; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; PEDF, pigment epithelium-derived factor; PKC, protein kinase C; rAAV, recombinant adeno-associated virus; sFlt-1, soluble Flt-1; TIMP, tissue inhibitors of metalloproteinase; VEGF, vascular endothelial growth factor.

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*This paper was presented at the GlaxoSmithKline/MRS Young Investigator session at the MRS Meeting, Royal College of Physicians, London, on 4 February, 2002.
Neovascularization is typically seen in ischaemic retinopathies and extends preretinally into the vitreous cavity. Neovascularization of the choroid is most commonly due to AMD and often extends subretinally.

elderly respectively [1–3]. Neovascularization in these conditions causes visual loss through increased vascular permeability leading to retinal oedema, vascular fragility resulting in haemorrhage, or fibrovascular proliferation with tractional and rhegmatogenous retinal detachment. Although neovascularization 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.

**NEOVASCULAR AGE-RELATED MACULAR DEGENERATION (AMD)**

AMD is the most common cause of blindness in industrialized countries, with an estimated incidence of 20,000 new cases annually in the U.K. and prevalence of 1.9% in people older than 50 years [7]. The pathogenesis of AMD is not well understood, but involves abnormalities of the extracellular matrix at the level of Bruch’s basement membrane [8]. Choroidal neovascularization in this context may be the result of hypoxia/ischaemia of overlying retinal pigment epithelial cells, due to either the thickening of Bruch’s membrane or abnormalities of choroidal perfusion [9–11], leading to the expression of pro-angiogenic factors to promote vascular proliferation.

**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 occlusion and ischaemia. The subsequent hypoxia-induced up-regulation of angiogenic growth factors results in neovascularization that extends from the inner retinal surface into the vitreous gel (Figure 1) [4]. Complications of neovascular proliferation are the major causes of persistent severe visual loss in diabetes through haemorrhage into the vitreous or retinal detachment (Figure 2) [5]. The current conventional treatment for established proliferative diabetic retinopathy is panretinal laser photocoagulation. This technique is able to induce regression of retinal neovascularization if initiated promptly, but is inherently destructive and is itself associated with significant predictable adverse effects on visual function [6]. Although ischaemia-induced retinal neovascularization is most commonly seen in diabetic eye disease, it is also a feature of many other retinopathies, including retinopathy of prematurity, retinal vascular occlusion, sickle cell disease and ocular inflammatory disorders.
Table 1  Experimental molecular therapies for ocular angiogenesis evaluated in clinical trials

<table>
<thead>
<tr>
<th>Condition</th>
<th>Agent</th>
<th>Mechanism of action</th>
<th>Route of administration</th>
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</thead>
<tbody>
<tr>
<td>Proliferative diabetic retinopathy</td>
<td>Sorbinil</td>
<td>Aldose reductase inhibitor</td>
<td>Oral dosing</td>
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<tr>
<td></td>
<td>LY333531</td>
<td>PKC/β-selective inhibitor</td>
<td>Oral dosing</td>
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<td></td>
<td>Octreotide</td>
<td>Somatostatin analogue; GH/IGF-1 inhibition</td>
<td>Repeated subcutaneous injections</td>
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<tr>
<td></td>
<td>Pegvisomant</td>
<td>GH-receptor antagonist</td>
<td>Repeated subcutaneous injections</td>
</tr>
<tr>
<td>Neovascular AMD</td>
<td>EYE001 (macugen)</td>
<td>VEGF inhibition by poly(ethylene glycol)ylated RNA aptamer</td>
<td>Repeated intravitreal injection</td>
</tr>
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<td></td>
<td>Rhino Fab V2 (AMD Fab)</td>
<td>VEGF inhibition by monoclonal antibody</td>
<td>Repeated intravitreal injection</td>
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<td></td>
<td>ADGVPEDF.11D</td>
<td>PEDF overexpression</td>
<td>Single intravitreal injection of Ad vector</td>
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<td></td>
<td>AG3340 (prinomastat)</td>
<td>Synthetic MMP</td>
<td>Oral dosing</td>
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<td></td>
<td>Anecortave acetate</td>
<td>Angiostatic steroid</td>
<td>Single pericocular injection</td>
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<tr>
<td></td>
<td>Triamcinolone acetone</td>
<td>Angiostatic steroid</td>
<td>Intravitreal injection</td>
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<td></td>
<td>Squalamine</td>
<td>Anti-angiogenic</td>
<td>Repeated intravenous injections</td>
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cytokines [12,13]. Exudation or haemorrhage from resulting neovascular complexes (Figures 1 and 2) that extend from the choroidal vasculature through breaks in Bruch’s membrane account for 80% of severe visual loss in this condition [14,15]. The currently accepted treatment modalities for choroidal neovascularization in AMD comprise thermal laser photocoagulation and photodynamic therapy. These ablative approaches can offer short-term benefit to certain subgroups of patients [16–18], but are associated with significant adverse effects [6,19]. Moreover, since they fail to address the underlying stimuli for blood vessel growth, these treatments are associated with high rates of persistent and recurrent disease [17,20] accompanied by an increased frequency of severe visual loss [21].

Pathological angiogenesis also occurs in tissues in the anterior segment of the eye. Neovascularization 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 the associated closure of the irido-corneal drainage angle, resulting in raised intraocular pressure and glaucoma. Neovascularization of the cornea can occur in response to a number of different insults, including trauma, infection, inflammation and contact lens wear [22]. This review, however, concentrates on 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 [22].

MOLECULAR BIOLOGY OF OCULAR ANGIOGENESIS

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. [23] 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 neovascularization in the eye. Cytokines that have been effectively targeted in experimental models include vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), pigment epithelium-derived factor (PEDF), matrix metalloproteinases (MMPs), angiotatin, endostatin, angiopoietin (Ang) and integrins. Additional targets specific to diabetic retinopathy include aldose reductase, advanced glycation end-products (AGEs), protein kinase C (PKC) and nuclear factor κB (NF-κB). Although there are, at present, no established molecular treatments for proliferative diabetic retinopathy or choroidal neovascularization, a number of experimental molecular therapies are currently being evaluated in clinical trials (Table 1).

VEGF is a potent endothelial-cell-specific mitogen that plays a critical role in angiogenesis [24,25]. VEGF is a 46 kDa homodimeric glycopeptide that is expressed by several different ocular cell types, including pigment epithelial cells, pericytes, vascular endothelial cells, neuroglia and ganglion cells [13,24,26,27], and in specific spatial and temporal patterns during retinal development [28]. Expression of VEGF is up-regulated by hypoxia in vitro [29] and in vivo [27] through a hypoxia-inducible factor-1 (‘HIF-1’) transcriptional element [30].
VEGF acts via specific receptors, fms-like tyrosine kinase (Flt)-1 and fetal liver kinase-1 (‘Flk-1’)/kinase insert domain-containing receptor (‘KDR’), which are high-affinity receptor tyrosine kinases expressed on vascular endothelial cells, resulting in the phosphorylation of proteins, including phospholipase Cγ, that lead to the formation of diacylglycerol (DAG), activation of PKC and, ultimately, to endothelial cell proliferation, migration, and increased vasopermeability. VEGF levels are increased in experimental models of retinal ischaemia [26,31], in patients with proliferative diabetic retinopathy [32–34], retinopathy of prematurity [35], retinal vein occlusion [33] and in choroidal neovascularization [36]. Placenta growth factor (‘PlGF’) is a member of the VEGF family that stimulates endothelial proliferation and angiogenesis in vitro and appears to potentiate the effect of VEGF either by enhancing its expression or by the formation of heterodimers [37]. 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 VEGF-receptor soluble Flt-1 (sFlt-1) has attracted particular attention for its potential therapeutic role in the control of neovascularization [38,39]. 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 kinase insert domain-containing receptor [40,41]. Other anti-VEGF strategies to control ocular neovascularization in experimental models have included neutralizing anti-VEGF monoclonal antibodies [42,43], soluble VEGF-receptor chimaeric proteins [44], oligonucleotides [45,46] and inhibition of a VEGF-specific protein kinase [25]. Two VEGF-directed therapies are currently under evaluation in clinical trials for choroidal neovascularization in AMD. EYE001 (macugen) is a VEGF antisense poly(ethylene glycol)ylated RNA aptamer (an oligonucleotide that acts like a high-affinity anti-VEGF antibody) [47] and RhuFab V2 (AMD Fab) is a monoclonal antibody fragment directed against VEGF [48]. Both these agents are administered by repeated intravitreal injection.

The cytokine IGF-1 mediates the mitogenic effect of growth hormone (GH). IGF-1 appears to play a permissive role in the development of ischaemia-induced retinal neovascularization and is able to induce retinal neovascularization directly [49]. Both local and systemic expression of IGF-1 appear to contribute to its intraocular levels [50]. IGF-1 inhibition by a receptor antagonist suppresses experimental retinal neovascularization [51]. Although a clinical trial of the GH-receptor antagonist pegvisomant has not demonstrated regression of diabetic retinopathy [52], trials of GH/IGF-1 axis inhibition using somatostatin analogues suggest that this approach can offer a beneficial effect in diabetic retinopathy [53,54] and may also be effective in choroidal neovascularization [55].

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 proteins [56] and was first described for its neurotrophic properties in vitro [57]. Subsequently found to protect against degeneration of photoreceptors [58,59] and ganglion cells [60], PEDF is also a potent inhibitor of angiogenesis, possibly through promotion of endothelial cells apoptosis [61]. It is down-regulated by hypoxia and its loss appears to play a permissive role in ischaemia-driven retinal neovascularization [62]. Tissue hypoxia induces an equilibrium shift between VEGF and PEDF and this imbalance has been proposed as a possible mechanism for the development of choroidal neovascularization in AMD [63]. The neuroprotective properties of PEDF make this a particularly attractive candidate for the control of neovascularization in age-related maculopathy, since degeneration of the retina is also a typical feature of this condition, and a clinical trial of PEDF for neovascular AMD has been proposed [64].

MMPs are a family of zinc-binding Ca 2+ -dependent neutral endopeptidases that function as proteolytic enzymes in the degradation processes of the extracellular matrix [65] and have important roles in development, wound healing and angiogenesis. Their inactive precursors are activated locally by proteolytic removal of N-terminal ends, and their activity is regulated further 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. MMP-1, -2 and -9 are believed to initiate and promote angiogenesis. MMP-2 expression by endothelial cells is up-regulated by hypoxia and VEGF and interacts with αβ3 integrin on endothelial cell surface to create localized areas of high proteolytic activity. MMP-2 and -9 preferentially degrade basement membrane components such as type IV collagen. Angiogenesis is disrupted by PEX, a non-catalytic C-terminal haemopexin domain of MMP-2 with integrin-binding activity [66,67]. MMP-7, -9 and -12 may block angiogenesis by converting plasminogen into angiotatin, which is one of the most potent angiogenesis antagonists [68]. TIMP-1, -2, -3 and possibly TIMP-4 inhibit neovascularization [69–71]. Critical roles for MMPs and TIMPs are implicated in ocular angiogenic disorders. MMP-2 and -9 are implicated in both retinal [72] and choroidal [73] neovascularization. TIMP-3 is synthesized by retinal pigment epithelial cells [74] 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 characterized by thickening of Bruch’s basement membrane and choroidal
neovascularization. Matrix degradation is an attractive target for angiostatic therapy, because it represents a critical step and final common pathway in angiogenesis. An oral synthetic MMP, AG3340 (prinomastat), has recently been the subject of evaluation in clinical trials for choroidal neovascularization in AMD.

Integrins are non-covalently associated transmembrane glycoprotein heterodimer receptors that mediate bidirectional interactions between extracellular matrix proteins and cytoskeleton across the plasma membrane and also function as signal transducers. The integrins $\alpha_v\beta_3$ and $\alpha_\beta_\gamma$ are implicated in angiogenesis [75]. Both are expressed in neovascular endothelial cells in proliferative diabetic retinopathy, whereas only $\alpha_v\beta_3$ is expressed in active choroidal neovascularization. Peptide antagonists administered systemically [76], locally [77] or by topical application [78] result in inhibition of experimental retinal angiogenesis, but these have yet to be evaluated in clinical trials.

Angiostatic steroids consist of glucocorticoids coupled to heparin. The mechanism of their angiostatic effect is believed to involve inhibition of MMPs and reduction in adhesion molecule expression [79], although 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 [80] and intravitreal injection of triamcinolone [81,82]. Squalamine is a novel anti-angiogenic amino sterol that acts, at least in part, by blocking mitogen-induced proliferation and migration of endothelial cells [83]. Administered systemically, squalamine significantly improves intracocular neovascularization in experimental models [84,85], and a clinical trial is currently underway to evaluate its effect in neovascular AMD.

A number of endogenous inhibitors of angiogenesis have been described. Angiostatin and endostatin, in particular, have yielded promising results in experimental models, although these inhibitors have yet to be evaluated in clinical trials for ocular neovascular disorders [86]. 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 stabilization and maturation of vessels through the action of an agonist ligand Ang1 and an antagonistic ligand Ang2. Ang1 mediates vessel maturation and remodelling and is essential for normal vascular development in the mouse [87]. 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 [88]. Ang1 also appears to confer resistance to VEGF-mediated vascular leakage [89]. Ang2 antagonizes the Tie2-receptor and its expression is up-regulated during physiological and pathological neovascularization [90]. sTie-2, a synthetic soluble receptor of Ang inhibits angiogenesis and has potential therapeutic value. Ang1 is also of potential therapeutic value for decreasing 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 [89].

In diabetic retinopathy, the mechanisms by which hyperglycaemia leads to the up-regulation of angiogenic growth factors (reviewed in detail elsewhere, see [91]) are complex and involve activation of the polyl pathway, non-enzymic glycation and oxidative stress; retinal hypoxia is the result of subsequent haemodynamic changes, microvascular occlusion and vascular cell apoptosis. This pathway offers a number of potential opportunities for specific molecular therapeutic intervention in proliferative diabetic retinopathy. Hyperglycaemia activates the polyl pathway in which glucose is converted into 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 [92], the effect of aldose reductase inhibitors in clinical trials of diabetic retinopathy is inconclusive [93]. Hyperglycaemia also leads to the irreversible formation of AGEs which, in turn, generate oxygen-derived free radicals that contribute towards increased oxidative stress and themselves increase autocrine retinal VEGF expression [94]. Inhibition of AGE formation is another attractive strategy that is currently being developed [95,96]. Activation of endothelial PKC by DAG, synthesized either de novo in chronic hyperglycaemia or via VEGF receptor activation, results in the up-regulation of platelet-activating factor expression with leucocyte activation, thrombus formation, haemodynamic changes and increased vascular cell permeability and proliferation. Interventions that increase DAG metabolism or inhibit PKC isoenzymes ameliorate the biochemical and functional consequences of DAG/PKC activation in experimental diabetes [97] and reduce the extent of ischaemia-induced experimental intracocular neovascularization [98]. Clinical trials to evaluate the effect of the PKC$\beta$-selective inhibitor LY335531 in diabetic retinopathy are in progress [99,100]. Death of retinal capillary cells can occur by apoptosis, especially in the context of fluctuating glucose levels; AGEs may induce activation of NF-$\kappa$B, a molecule that appears to be an important signalling molecule in retinal vascular cell survival [96,101]. Inhibition of NF-$\kappa$B activation offers a potential strategy to prevent retinal hypoxia through the inhibition of vascular cell apoptosis [101].

It is clear that angiogenesis is a highly complex process and that the effects of cytokines on endothelial cell-matrix interactions can be contextual [102]. 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 growth [103],
the introduction of a single agent to tip the balance towards angiostasis may be all that is required to achieve a therapeutic effect. As synergistic effects of angiostatic proteins have been described both in vitro and in vivo [104], a combination of therapeutic factors may result in a particularly powerful effect.

**RATIONALE FOR OCULAR GENE TRANSFER OF ANGIOSTATIC PROTEINS**

Although 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 revascularization 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 and 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 thromboembolic events [105]. In addition to these concerns, the production of recombinant proteins is technically difficult and their manufacture is expensive [86]. 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 neovascularization in experimental models is decreased by the repeated intravitreal injections of neutralizing anti-VEGF monoclonal antibodies [42], recombinant soluble VEGF-receptor chimaeric proteins [44] and antisense oligodeoxynucleotides [45]. 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 levels [106] 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 localized, 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 minimizing 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.

**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 compartmentalized and readily accessible by microsurgical techniques under direct visualization. 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 immune privilege of many ocular tissues appears to confer an advantage in terms of long-term transgene expression [107]. The optical transparency of the eye enables green fluorescent protein ('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.

Ocular gene transfer strategies have been developed for gene replacement in recessively inherited disorders [108] and gene inactivation in dominantly inherited disorders [109]. As a model system for the development of gene therapy for inherited disorders, the eye offers the additional advantage that inherited retinal degenerations are relatively common and well-described [110]. Suicide-gene strategies have been used to target proliferative diseases such as retinoblastoma [111] and proliferative vitreoretinopathy [112]. Gene transfer also offers an attractive strategy for the local delivery of small molecule therapeutic proteins in a broad range of complex acquired disorders. These agents include neuroprotective and anti-apoptotic factors for degenerative disease, immunomodulatory factors for immune disorders and angiostatic proteins for neovascular disorders.

A number of different viral and non-viral vector systems for gene transfer to ocular tissues have been extensively evaluated [113,114]. 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. 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 with viral vectors [115]. For efficient sustained transduction of photoreceptor cells, recombinant adeno-associated virus (rAAV) vectors are currently the vector of choice [116–118]. Lentiviral vectors, on the other hand, stably transduce retinal pigment epithelial cells, and are much less efficient than rAAV at transducing photoreceptors [119,120]. Adenoviral (Ad) vectors efficiently target
cells of the outer retina [121,122], but their duration of expression is limited by immune responses to the vector [123]. Retroviral vectors specifically transduce dividing cells and have been employed for suicide-gene approaches to proliferative and neoplastic intraocular disorders [111,124]. Since ocular tissues are highly compartmentalized, the pattern of tissue transfection by a given vector is also dependent on the site of its intraocular administration. Delivery of rAAV 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 in the inner retina, at least in the fully developed retina [125]. For these reasons the optimal choice of vector and route of delivery for any given application depends on the cell to be targeted and the desired time course of transgene expression.

ANGIOSTATIC GENE TRANSFER IN EXPERIMENTAL RETINAL NEOVASCULARIZATION

Although retinal neovascularization is not a prominent feature in experimental models of spontaneous or streptozotocin-induced diabetes, it can be readily quantified in rodent models of ischaemia-induced retinopathy, and these are extensively utilized for the evaluation of novel angiostatic approaches. Murine oxygen-induced retinopathy is a highly reproducible model of ischaemia-induced VEGF-dependent [26] retinal neovascularization [126]. Exposure of mouse pups to 75% oxygen for 5 days causes extensive retinal capillary closure. On their subsequent return to room air, the relative hypoxia results in retinal ischaemia and VEGF-dependent preretinal neovascularization in 100% of animals. The neovascular response is demonstrable by angiography and quantified histologically by counting the number of neovascular endothelial cells projecting from the retina into the vitreous. We have shown in this model [127] that inhibition of VEGF by local gene transfer of its soluble receptor sFlt-1 consistently reduces neovascularization by 50%. These findings are consistent with other studies of VEGF inhibition in the same model using repeated intravitreal injection of recombinant sFlt-1 [44] or antisense oligodeoxynucleotides [45]. It is unclear whether residual neovascularization is due to incomplete inhibition of the VEGF response or, rather, the result of the uninhibited activity of an alternative angiogenic pathway. The ability of a VEGF-receptor kinase inhibitor to completely prevent neovascularization when delivered systemically in the same model suggests that improved gene delivery and expression might be expected to overcome this limitation [25].

Both Ad- and rAAV-mediated expression of sFlt-1 resulted in similar angiostatic efficacy, despite contrasting spatial patterns of gene transfer and expression profiles. Intravitreal injection of Ad vectors results in transduction of tissues in the anterior segment, but not the retina [127–129]. However, the efficacy of Ad vectors in decreasing retinal neovascularization suggests that the active protein 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 rAAV almost exclusively results in transduction of cells in the inner retina [127]. 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 localization of gene expression close to the site of pathology and the known biological characteristics of gene transfer by rAAV vectors makes this a highly attractive strategy for sustained therapy of retinal neovascularization.

Neovascularization in the murine model of ischaemia-induced retinopathy occurs as a short-lived response; after post-natal day 21 no further proliferation occurs and the new vessels regress spontaneously [126]. Even in this model in which the neovascular response is relatively transient, delivery of sFlt-1 by gene transfer after a single procedure is at least as effective as repeated intravitreal injection. The advantage of a gene transfer approach in facilitating sustained inhibition is likely to be of particular relevance when applied to conditions in which there is a longer-term predilection to neovascularization. Although rAAV-mediated expression of sFlt-1 is likely to be sustained, the long-term effect on neovascularization cannot be confirmed in this model and will need to be evaluated in further appropriate models and clinical studies.

Loss of vision in diabetic retinopathy can be the result of macular oedema as well as neovascularization. In addition to its central role in angiogenesis [41], VEGF promotes vascular permeability through Flt-1 receptors [130]. By reducing vasopermeability, VEGF antagonists such as sFlt-1 may have additional potential applications in the management of macular oedema, a common cause of visual loss in diabetes [131], uveitis [132] and following cataract surgery [133].

ANGIOSTATIC GENE TRANSFER IN EXPERIMENTAL CHOROIDAL NEOVASCULARIZATION

Although an ideal experimental animal model of AMD is currently lacking, choroidal neovascularization can be induced by laser-induced rupture of Bruch’s basement membrane [134] or the local delivery of pro-angiogenic cytokines, including VEGF [135,136] and basic fibroblast growth factor (‘bFGF’) [137]. 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 quantified be by in vivo fluorescein angiography.

rAAV vectors are excellent candidates for gene transfer in the treatment of choroidal neovascularization, since they efficiently and stably transduce photoreceptors and retinal pigment epithelial cells which are both in close proximity to the developing neovascular complex. We have found that local rAAV-mediated gene transfer of the VEGF inhibitor sFlt-1 by local subretinal vector delivery prior to laser-rupture results in a significant reduction in the extent of the subsequent neovascular response by up to 40% (J.W.B. Bainbridge, A. Mistry, A.J. Thrasher and R.R. Ali, unpublished work). Subretinal delivery of a similar vector distant to the site of laser-rupture results in a more modest angiostatic effect [138]. rAAV vectors have been used to effectively suppress choroidal neovascularization by expression of other anti-angiogenic factors, including PEDF [139] and angiotatin [140]. rAAV-mediated gene transfer of PEDF not only reduces the progression of laser-induced choroidal neovascularization, but also accelerates its spontaneous regression [141].

Alternative vectors for gene therapy of choroidal neovascularization include retroviral and lentiviral systems. Retroviral vectors specifically target proliferating cells at sites of laser injury [142,143], but this strategy does not address the underlying angiogenic process and is not likely to offer a sustained therapeutic effect. Lentiviral vectors are attractive candidates for their ability to stably transduce non-dividing cells and their relative specificity for retinal pigment epithelial cells following subretinal delivery [119,120].

REGULATION OF ANGIOSTATIC TRANSGENE EXPRESSION

Many molecular mediators of angiogenesis mediate essential physiological functions in the retina. VEGF itself is believed to mediate important housekeeping functions in the mature retina and acts as a survival factor for vascular endothelium [144]. Although we have detected no evidence that VEGF inhibition by sFlt-1 gene transfer has an adverse effect on normal retinal vascular development or structure [127], there remains the possibility that its long-term uncontrolled inhibition by the sustained and unregulated expression of angiostatic proteins presents a risk of adverse local effects. The development of strategies to achieve effective targeting of expression and to enable appropriate regulation of gene expression is therefore desirable to minimize the potential for local toxicity.

One approach to regulation of gene expression is through the incorporation of inducible promoters responsive to the administration of exogenous pharmaco- logical agents. Gene expression mediated by rAAV vectors in the retina can be regulated by tetracycline-inducible [145] or rapamycin-inducible systems [146]. Pharmacological approaches such as these are dependent, however, on regular clinical observation to determine appropriate dosing and are limited by inefficient drug penetration across the blood–retinal barrier. An alternative approach is use of a tissue-responsive promoter to drive transgene expression according to specific features of the local tissue environment. Since retinal neovascularization is typically the result of local tissue hypoxia, the hypoxia-regulated expression of angiostatic molecules is an attractive strategy. The incorporation of a hypoxia-response element (HRE) into promoter sequences of therapeutic constructs results in the regulation of transgene expression in response to local tissue hypoxia. An HRE is a specific enhancer present in a number of genes, including the gene encoding VEGF [147], and mediates transcriptional responses to hypoxia through binding of activated hypoxia-inducible factor-1. Hypoxia-regulated gene expression has been used for targeting of tumours [148,149] and myocardial ischaemia [150], and to achieve physiological regulation of erythropoietin expression in experimental anaemia [151].

In experimental retinal and choroidal neovascularization, the incorporation of an HRE into the promoter sequence targets reporter gene expression to sites of neovascularization and results in expression that is not sustained beyond the period of active angiogenesis [152]. This system offers an attractive strategy to achieve spatial and temporal regulation of expression of angiostatic proteins in the management of retinal and choroidal neovascularization by gene transfer. In this way, sites of active neovascularization may be effectively targeted, while minimizing inappropriate expression elsewhere or during periods of angiogenic inactivity. Moreover, this system is ‘vigilant’ in that HRE-driven expression may anticipate the onset of a neovascular response to ischaemia in a way not possible with pharmacologically inducible systems, where dosing is dependent on the clinical observation of established new vessels. This strategy might also offer 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.

PRECLINICAL STUDIES OF RETINAL GENE TRANSFER

Although rAAV vectors are well characterized in terms of retinal gene transfer in rodent [125,153,154], a requisite to their clinical application in patients is the thorough evaluation of their efficacy and safety in intermediate animal models, such as dogs and primates.
The larger eye facilitates a more relevant surgical approach to the delivery of vector suspension to the subretinal space, and larger animals are more amenable to sophisticated assessments of visual function, such as behavioural testing, and their longevity enables the long-term evaluation of the effects of interventions.

There have been few reports of rAAV-mediated gene transfer in large animals [118,135,155,156]. We have demonstrated [156a] that subretinal delivery of rAAV-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 subretinal delivery and is sustained in cells at this site for at least 18 months. Reflux of vector suspension into the vitreous cavity can result in transfection of cells in the inner retina [118,155] and even in the anterior segment of the eye. For clinical applications, the restriction of gene expression to target cells by means of tissue-specific promoters may be desirable to minimize adverse effects resulting from inappropriate expression of therapeutic genes in non-target cells. Although immune responses to rAAV-vector antigens have not been reported, there is a strong suggestion in some cases of an immune response to the expressed reporter gene green fluorescent protein. Although this finding may not be directly relevant to the delivery of therapeutic proteins, the possibility that similar immune responses might occur is an important concern. Such a response may not only neutralize the therapeutic effect of the expressed protein, but also induce intraocular inflammation.

Very few studies have evaluated the functional effects of rAAV vector delivery to the retina in normal animals. Although subretinal delivery of rAAV vectors in primates resulted in no apparent adverse effect on retinal function [118], we have detected a modest attenuation in global retinal function in dogs, as demonstrated by electoretinography [156a]. A similar effect following subretinal delivery of recombinant feline immunodeficiency virus vectors in primates has been described [120]. This attenuation of retinal function following subretinal vector delivery is consistent with the effect of short-term detachment of the neurosensory retina [157–159]. Modifications of surgical techniques or the co-administration of neuroprotective agents may protect the retina against such detachment-induced functional attenuation. In the clinical context, the beneficial effect of an expressed therapeutic transgene may more than compensate for a modest delivery-related attenuation in retinal function.

**FUTURE DIRECTIONS**

There is now extensive evidence to support the efficacy of an in vivo gene therapy approach in rodent models of ocular neovascularization and there is accumulating data on the safety of rAAV and lentiviral vectors in normal large animals. Preclinical studies using rAAV vectors in the treatment of a variety of inherited monogenic defects and acquired diseases have been performed. rAAV vectors are particularly attractive as gene transfer vectors to the retina, as they efficiently and stably transfect postmitotic photoreceptors and retinal pigment epithelial cells, they have no known pathogenicity in humans and are minimally immunogenic. Recent improvements in rAAV packaging should allow the generation of sufficient quantities of vector for clinical trials. The small rAAV genome that can be packaged, however, restricts its use to the transfer of smaller genes. Recombinant rAAV vectors, unlike the wild-type virus, do not appear to integrate site-specifically into the mammalian genome and, although they may exist as large episomal concatamers (as has been observed in other tissues) for long periods of time, the possibility of insertional mutagenesis is a genuine concern [160]. Attempts to develop the means to achieve targeted site-specific integration, however, may offer a potential advantage. In clinical trials of rAAV-mediated gene therapy for cystic fibrosis [161] and haemophilia [162,163], there is little or no evidence of toxicity associated with vector administration or transgene expression, no germine transmission of vector sequences and no induction of inhibitory antibodies against the expressed protein. However, for both these applications, there has also been little evidence for efficient gene transfer, and toxicity may only be revealed when this is improved. There is some evidence to support the efficiency and safety of lentiviral vectors for retinal gene therapy [119,120]. Although the use of HIV 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 [164]. The use of lentiviral vectors, such as feline immunodeficiency virus and equine infectious anaemia virus, that are non-pathogenic to humans may offer an even safer alternative.

There are a number of promising angiostatic proteins that might be effective in a clinical trial of gene therapy for ocular neovascularization. The central role of VEGF and the proven efficacy of VEGF-directed approaches in experimental models make this factor a particularly attractive target. Ongoing clinical trials of the anti-VEGF monoclonal antibody and the VEGF aptamer in neovascular AMD will offer invaluable data regarding the potential efficacy and toxicity of a gene transfer approach. PEDF is another attractive candidate for the control of neovascularization particularly in AMD, since its neuroprotective properties may slow the degenerative features of this condition, and a phase I clinical trial of Ad-mediated overexpression of PEDF for choroidal neovascularization in AMD has been proposed.
Potential clinical strategy for the hypoxia-retina. Gene targeting of NF-κB may help to prevent retinal hypoxia through the inhibition of vascular cell apoptosis. Nitric oxide has a key role in the control of physiological and pathological angiogenesis [165]. Inducible nitric oxide synthase (iNOS) is expressed in the ischaemic retina, plays a crucial role in retinal neovascular disease and offers a very attractive novel strategy for the control of pathological retinal angiogenesis through the promotion of appropriate revascularization of hypoxic retina [166].

Conventional systemic delivery of an appropriate agent may be appropriate when therapeutic intraocular levels can be achieved without the risk of significant adverse systemic effects. Such an approach is realistic where the agent has good intraocular penetration or its effect is specific to the retina. Oral administration of the PKCβ isoenzyme-selective inhibitor LY333531, for example, may offer a valuable therapeutic effect in the retinal without concomitant systemic adverse effects. If only a short-lived effect is required, then a single intraocular injection of recombinant protein may be adequate, but this seems unlikely to be effective in ocular angiogenic disorders that have a prolonged course. A gene transfer approach offers a particular advantage where the sustained, targeted and regulatable local expression of a powerful angiostatic protein minimizes the risk of systemic adverse effects or obviates the need for repeated intraocular delivery procedures.

The possibility of local and systemic adverse effects, due to expression of a given angiostatic factor, will require careful investigation prior to its use in patients. To date, there is minimal experience of therapeutic gene transfer in large animal models of retinal or choroidal neovascularization and evidence of efficacy and toxicity of specific angiostatic proteins in these models is desirable before embarking on clinical trials in patients. Established models in primates include ischaemia-induced iris neovascularization following laser retinal vein occlusion [31] and laser-induced choroidal neovascularization [134]. Although these models are valuable, the neovascularization in each case occurs in response to a single insult and is relatively short-lived. 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. The incorporation of regulatable, pharmacological or tissue-responsive elements into the vector construct would add additional safeguards against the possibility of adverse effects due to inappropriate transgene expression.

For the first human trials of ocular angiostatic gene transfer, the clinical indications must be carefully chosen in order to optimize 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 neovascularization. Although angiostatic gene transfer may offer a safer alternative, this novel approach might be most appropriately evaluated in the first instance in patients with neovascular AMD, where there is no effective treatment currently available. One possible approach in this condition might be a single subretinal administration of a rAAV vector expressing the VEGF inhibitor sFlt-1 (Figure 3). Should hypoxia be confirmed as an important factor in the pathogenesis of neovascular AMD, then the incorporation of a hypoxia-responsive promoter could provide a valuable means to restrict expression to the site and period of active angiogenic drive. An alternative strategy for neovascular AMD proposed recently [64] involves the intravitreal administration of an Ad vector expressing PEDF. Although angiostatic gene transfer may well offer an effective treatment used in isolation, it may also have...
a valuable role as an adjunct to established treatments or other experimental approaches.

Recent successes in clinical trials of gene therapy [167] 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, a child effectively cured of X-linked severe combined immunodeficiency (‘X-SCID’) by retrovirus-mediated gene transfer to bone marrow cells, developed a lymphoproliferation that evolved into T-cell leukaemia. Although the molecular pathogenesis of this process is not yet fully understood, the insertion of the transgene into a recognized 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 Gene Therapy Advisory Committee [168] 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. Although ocular neovascular disorders are not life threatening, the impact of blindness on quality of life should not be underestimated. Although 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.

ACKNOWLEDGMENTS

J.W.B.B. is a Wellcome Trust Research Training Fellow. This work was also supported by Diabetes UK, Fight for Sight, the British Retinitis Pigmentosa Society, and the Sir Jules Thorn Charitable Trust. Figure 2(c) was kindly provided by Samantha Dandekar, Moorfields Eye Hospital, London, U.K.

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Received 5 November 2002/13 January 2003; accepted 5 February 2003
Published as Immediate Publication 5 February 2003, DOI 10.1042/CS20020314