Gene Ther Mol Biol Vol 7, 135-151, 2003

Gene therapy for vascular diseases

Review Article

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Key words: Atherosclerosis, gene therapy, adenoviruses, vascular diseases.

Received: 2 July 2003; Accepted: 18 July 2003; electronically published: July 2003

Summary

Currently, successful pharmacological treatments are unavailable for many vascular diseases. Many patients undergo surgical interventions and then present with recurrence of symptoms. Recently, gene therapy using both non-viral and viral delivery has emerged as a novel tool to treat patients with vascular diseases. Here we discuss the requirement to develop suitable gene delivery vectors for vascular diseases. Our expanding knowledge of the pathogenesis of vascular diseases has allowed the identification of several gene therapy strategies and many candidate genes. Gene therapy using both gene knockout and gene overexpression has been considered. In pre-clinical studies, antisense and decoy oligonucleotides have been successfully employed to knockout the expression of stimulatory genes such as cell cycle promoters and growth factors. Furthermore, overexpression of inhibitory genes such as cell cycle inhibitors and nitric oxide and overexpression of genes to promote therapeutic angiogenesis have been shown potential in animal models. The progress of pre-clinical studies to treat vein graft failure, restenosis, myocardial and peripheral ischemia and hypertension and the development of clinical trials will be discussed. Despite the quite promising findings with clinical trials, particularly with therapeutic angiogenesis, improved gene transfer vectors and methods for safe long-term gene transfer are still required to bring gene therapy to clinical practice.

I. Introduction

Gene therapeutics have been proposed as a potential novel therapy for a host of diverse disease that encompass acquired conditions such as cancer, cardiovascular disease and arthritis as well as monogenic diseases through gene replacement strategies. In theory the concept has seemed relatively simply; in practice, however, gene therapy is extremely complex, both technically and clinically. It requires a multifaceted approach involving identification of suitable therapeutic gene(s), identification of a suitable gene delivery vehicle together with the availability of satisfactory pre-clinical models in which to evaluate the potential benefit of the gene therapeutic approach, particularly against alternative pharmacological therapies, if available. The issue of long-term safety of gene therapy approaches is still unclear. To date, major progress at the clinical level has been made in defined areas, particular cancer, cystic fibrosis, haemophilia and some vascular diseases. These advances have not been without major drawbacks. Tragic events involving high dose delivery of adenoviral vectors to a patient on a gene therapy clinical trial for ornithine transcarbamylase (OTC) deficiency as well as the evolution of leukaemia in severe combined immunodeficiency (SCID) patients involving retroviral vectors (Cavazzana-C et al, 2000; Somia et al, 2000; Fox 2003) have highlighted safety issues relating to gene delivery vectors. In vascular diseases, successful gene therapy will require the following:

Identification of the optimal transgene cassette. Expression systems vary considerably for different gene therapy applications. Traditionally strong viral promoters have been used to provide maximal levels of expression in a multitude of recipient cell types. However, it is becoming increasing important to supply expression selectively to individual cell types or in a regulated manner through inducible promoters (such as tetracyclin system (Gossen et al, 1992; Vigna et al, 2002) thus circumventing potentially deleterious effects of transgene expression in non-target cell types. Additionally, viral promoters, particularly the cytomegalovirus immediate

early promoter (CMV IEP) is prone to host-mediated silencing in vivo (De Geest et al, 2000) leading to a shut down in transgene expression, an effect not observed with cell-specific promoters. Further optimisation of expression cassettes can be made through incorporation of introns and enhancers to elevate promoter activity as well as post-transcriptional modifications including the Woodchuck post-transcriptional regulatory element (WPRE) which is thought to act through promoting mRNA stability (Loeb et al, 1999; Zufferey et al, 1999).

Optimisation and evaluation of the gene delivery vehicle. At present the repertoire of gene delivery vectors available for human gene therapy is limited. Traditionally, non-viral vectors such as naked DNA and liposome DNA complexes provide low efficiency gene transfer and are restricted to the delivery of highly potent biological agents, such as angiogenic gene therapy (see below). Improvements in the efficiency of non-viral vectors, such as inclusion of targeting peptides into DNA liposome complexes (Hart et al, 1997; Parkes et al, 2002) have been realised but are still someway from the efficiency of viral vectors. Certain viruses, by virtue of evolution, infect human cells with high efficiency resulting in high potency gene transfer and overexpression of candidate therapeutic genes. For gene delivery to vascular tissues the current armoury of viral vectors includes adenoviruses (Ad), adeno-associated viruses (AAV), lentiviruses and Semliki forest viruses.

Efficient modalities for gene delivery to the target site. Certain vascular diseases, such as vein grafting are optimal for gene therapyapy since the target tissue (i.e. the vein to be grafted) is harvested and is available ex vivo for gene delivery prior to grafting within a clinically relevant time window (approximately 30 minutes). This enables delivery of genes in a safe and efficient manner (Baker et al, 1997; Tamirisa et al, 2002). Due to the short time frame, however, efficient vectors are required. Adenoviral vectors have proven particularly suited for this application (Channon et al, 1997; George et al, 2000; Tamirisa et al, 2002). Conversely, gene delivery to blood vessels in vivo requires the use of devices to allow localised in vivo gene delivery. Specific catheter systems have been developed and utilised with high efficiency for post-angioplasty and in-stent restenosis in a variety of animal species and blood vessels (French et al, 1994; Klugherz et al, 2000, 2002). Additionally, local delivery technology has been applied for gene therapyapy aimed at the myocardium. Different applications, such as atherosclerosis or hypertension require alternate delivery systems and often rely on intravenous vehicle administration.

Together, a combined approach to optimise the gene expression system, the delivery vehicle and the route of delivery are required for successful gene therapy. A number of key areas within vascular diseases have successfully exploited this and advanced to clinical trials while other areas have been severely limited due to deficiencies in one or more of the above requirements. Here, we discuss a number of these applications.

There is no doubt that gene therapy may offer advantages above traditional pharmacological therapies in certain respects. Delivery of gene can be achieved locally in the vasculature thereby increasing the selectivity and, potentially, the safety. This would be particularly important when the therapy may have an adverse effect if contact to non-target tissue in vivo occurred. Since many of the strategies that have been designed to be effective in vascular disease may be deleterious if exposed to non-target tissue, this advantage becomes very important. For example, in development of gene therapy for vein graft failure (see later) pro-apoptotic genes are highly effective but clearly their expression in other tissues such as the liver, may be detrimental. Likewise, in restenosis post-angioplasty (cytotoxic or cytostatic strategies) and angiogenesis gene therapy can be delivered locally and is a pre-requisite for clinical translation. A second (and equally important) advantage of gene therapy might be the requirement for only a single administration compared to the requirement for multiple administrations of conventional drugs, often daily for the lifetime of the patient. Again, this depends largely on the application and is to date unproven. Evidence suggests that beneficial effects of gene therapy for hypertension, vein grafting and restenosis can be elicited in the long term from single administrations (see later). This provides ample pre-clinical evidence to support these concepts.

In the following review, we discuss gene therapy for some vascular diseases and its progression in different experimental and clinical applications.

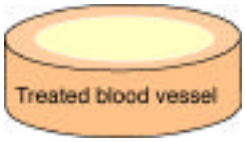
II. Local gene delivery to the vessel wall

It has been known for over a decade that gene delivery to the vessel wall can result in alterations in cell behaviour (Nabel et al, 1993 a, b, c) thereby initiating a plethora of studies that have evaluated and optimised gene delivery to the vessel wall. Although the first studies revealed that non-viral gene delivery could lead to phenotypic modulation of cell behaviour, it soon became clear that adenoviral vectors provided the most efficient means to achieve high-level gene delivery to the vessel wall in vivo (Lemarchand et al, 1993; French et al, 1994). Pioneering studies by Lemerchand and colleagues (1993) and French et al, (1994) showed that local exposure of high titre adenoviral vectors to normal and diseased blood vessels in vivo led to high-level transduction, in sheep and rabbit models, respectively. Catheter systems were rapidly developed and optimised for gene delivery post-angioplasty resulting in transgene expression throughout the vessel wall in a geographical localisation defined by the mode of vector delivery by the catheter utilised. This initiated a host of studies and led to the use of adenoviral vectors as the most commonly used modality through which to deliver genes to the vessel wall in vivo. However, this is not without limitations since adenoviral-mediated gene delivery was found to evoke an inflammatory response in the vessel wall leading to toxicity and endothelial cell activation (Newman et al, 1995). Furthermore, the use of these first-generation Ad vectors only resulted in transient gene expression lasting 7-14 days. Unlike other tissues, second generation vectors (that contained modifications of the Ad genome to reduce

expression of Ad-related genes) did not lead to sustained transgene expression in the vessel wall in vivo (Engelhardt et al, 1994; Wen et al, 2000). Other vector systems have recently been tested including improved non-viral systems such as peptide-targeted DNA/liposome complexes (Hart et al, 1997; Parkes et al, 2002), HVJ-modified liposomes (Morishita et al, 1995; Von Der Leyen et al, 1995; Dzau et al, 1996) and ultrasound-enhanced systems (Lawrie et al, 1999; Taniyama et al, 2002). Likewise, other viral vectors (including adeno-associated viruses (Maeda et al, 1997; Richter et al, 2000), Semliki-forest viruses (Lundstrom et al, 2001) and lentiviruses (Dishart et al, 2003) have been utilised. Modified viral systems in particular provide opportunities to modify the longevity of transgene expression as well as the principle cell type transduced. As an example, adeno-associated viruses (AAV) transduce smooth muscle cells in the vessel wall, even in the presence of an intact endothelial layer (Richter et al, 2000). This is in direct comparison to Ad-mediate delivery since endothelial transduction is high when an intact endothelium is present and represents a barrier to transduction (Lemarchand et al, 1993). This finding may in part be due to different physical sizes of Ad and AAV and due to different vector tropisms of each, which is dictated by host expression of viral receptors and co-receptors (Wickham et al, 1993; Bergelson et al, 1997; Tomko et al, 1997; Summerford et al, 1998; Qing et al, 1999; Summerford et al, 1999; Dishart et al, 2003). Hence, these systems have provided researchers with a diverse range of vectors through which to evaluate the phenotypic effects of overexpression of candidate therapeutic genes in the vessel wall in vivo.

III. Gene therapy and vein graft failure

The failure of vein bypass grafts in the coronary or lower extremity circulation is a common clinical occurrence that incurs significant morbidity and mortality. Despite the very common use of saphenous vein grafts to treat coronary and lower extremity occlusions the failure rate is extremely high, approximately 50% and 70% of vein grafts fail within 5-10 years after surgery, respectively (Angelini 1992; Conte et al, 2001). To date, pharmacological approaches to prolong vein graft patency have produced very limited results. Consequently, genetic approaches to modulate bypass grafts are actively being studied both in vitro and in vivo and are progressing to clinical trials. Vein grafts are uniquely amenable to intraoperative genetic modification because of the ability to manipulate the tissue ex vivo with controlled conditions. We will describe how both gene overexpression and gene blockade strategies have been tested, and how the latter is now in clinical trials (see also **Figure 1** for schematic summary of gene therapy strategies).



Restenosis

Vein Graft Failure

Angioplasty Intimal proliferation Constrictive remodelling	Stent Placement Intimal proliferation	Early Failure Thrombosis	Late Failure Intimal proliferation Constrictive remodelling
			
Gene Therapy Strategies ●*Anti-VSMC proliferation:* ●-Cytostatic: cell cycle inhibitors, antisense cell cycle genes ●& growth factors ●-Cytotoxic: tk, p53, ●*Anti-thrombotic:* uPA, tPA, NO ●*Re-endothelialization:* VEGF* ●*Anti-VSMC migration & matrix remodelling:* TIMPs	Gene Therapy Strategies ●*Anti-VSMC proliferation:* ●-Cytostatic: cell cycle inhibitors, antisense cell cycle genes ●& growth factors ●Cytotoxic: tk, p53, ●*Anti-thrombotic:* uPA, tPA, NO ●*Re-endothelialization:* VEGF	Gene Therapy Strategies ●*Anti-thrombotic: none tested* ●*Re-endothelialization:* C-type natriuretic peptide	Gene Therapy Strategies ●*Anti-VSMC proliferation:* ●-Cytostatic: cell cycle inhibitors, antisense cell cycle genes, transcription factors (E2F)* & growth factors ●*Re-endothelialization:* VEGF* ●*Anti-VSMC migration & matrix remodelling:* TIMPs

Figure 1: Gene therapy strategies for the treatment of restenosis and vein graft failure. Many preclinical studies have been utilised to determine the potential of these various strategies * indicates those that have progressed to clinical trials.

A. Biological processes involved in restenosis and molecular targets in vein graft failure

A complex series of biological events is initiated in the vein immediately after implantation into the arterial circulation. Within the first few days after implantation many vein grafts fail due to thrombosis, stimulated by endothelial injury (Bryan et al, 1994). Furthermore, in the first 24 hours vein grafts undergo a period of ischemia followed by reperfusion, which leads to the generation of superoxide and other reactive oxygen species that triggers cytoxicity of endothelial and smooth muscle cells (Shi et al, 2001; West et al, 2001). The grafted vein is then targeted by an acute inflammatory response involving neutrophil and mononuclear cell recruitment and oxidative stress persists (West et al, 2001). In the first week after implantation matrix remodelling and migration of smooth muscle cells into the intima takes place; once in the intima the smooth muscle cells proliferate contributing further to the intimal thickening (Newby et al, 1996). Each of these processes offers a set of potential molecular targets for gene therapyapy.

B. Anti-thrombotic and accelerated re-endothelialization strategies

Anti-thrombotic strategies have been investigated as a relevant target for gene transfer to reduce thrombosis in various models of arterial injury and thrombosis formation. Thrombosis is dramatically reduced using natural anti-thrombotic, anti-aggregatory, and fibrinolytic pathways such as overexpression of thrombomodulin (Waugh et al, 1999), tissue factor pathway inhibitor (Nishida et al, 1999; Zoldhelyi et al, 2000), CD39 (Gangadharan et al, 2001) and tissue plasminogen activator (Waugh et al, 1999). Despite their proven success, the potential of these anti-thrombotic strategies has not been widely tested in vein graft models perhaps due to the availability of pharmacological treatments. However, acceleration of re-endothelialization by gene transfer of C-type natriuretic peptide in rabbit jugular vein grafts reduced both thrombosis and intimal thickening (Ohno et al, 2002). This illustrates that promoting re-endothelialization and reducing thrombosis is a promising strategy to circumvent vein graft failure.

C. Anti-proliferative strategy

In an attempt to inhibit VSMC proliferation in vein grafts both overexpression of cell cycle inhibitory proteins and inhibition of cell cycle promontory genes using antisense has been investigated in arterial injury and vein graft models. In fact it is thought that strategies targeting multiple cell cycle genes offer greater potential than single targets. Rabbit vein grafts treated simultaneously with antisense oligonucleotides to proliferating cell nuclear antigen (PCNA) and cell division cycle-2 kinase showed reduced intimal thickening and diet induced atherosclerosis (Mann et al, 1995).

Recently, transfection of cis-element double-stranded oligonucleotides (decoy ODNs) has been reported as a new powerful tool in a new class of anti-gene strategies for gene therapyapy. Transfection of double-stranded ODN corresponding to the cis sequence will result in attenuation of the authentic cis-trans interaction, leading to removal of trans-factors from the endogenous cis-elements with subsequent modulation of gene expression. A decoy to E2F, which induces the coordinated expression of a number of critical cell cycle genes, including PCNA, cyclin-dependent kinase-1, cell division cycle-2 kinase, c-myc, c-myb, was used successfully. This E2F decoy ODN not only almost completely inhibited intimal thickening after balloon injury of the rat carotid at two weeks after injury (Morishita et al, 1995), but sustained inhibition was observed after eight weeks. This inhibition of intimal thickening was also observed using a porcine coronary artery model (Nakamura et al, 2002). Furthermore, a single intraoperative pressure-mediated delivery of E2F decoy effectively provided vein grafts with long-term (up to 6 months) resistance to intimal thickening and atherosclerosis (Ehsan et al, 2001). Interestingly, it has been demonstrated that although E2F decoy ODN treatment of vascular grafts inhibits VSMC proliferation and activation, it spares the endothelium, thereby allowing normal endothelial healing (Ehsan et al, 2002). A clinical trial (PREVENT) using intraoperative delivery of E2F decoy ODN to infrainguinal arterial bypass grafts demonstrated fewer graft occlusions, revisions, or critical stenoses in the E2F-treated group (Mann et al, 1999). Recently, a corporate-sponsored (Corgentech, Inc, Palo Alto, Calif) phase II trial of E2F decoy treatment of coronary vein grafts was completed (SoRelle 2001). This study, which involved 200 patients revealed a 30% reduction in critical stenosis and has formed the basis for design of a phase III trial in coronary bypass grafting. Furthermore, on the basis of this combination of preclinical and phase I/II clinical data, a phase III trial of E2F decoy ODN for the prevention of lower extremity vein graft failure involving 1400 patients was initiated in December 2001.

D. Pro-apoptotic strategy

In addition to the above-mentioned cytostatic approaches, cytotoxic strategies have also been considered. Delivery of TIMP-3, which in addition to inhibiting MMP activity and VSMC migration promotes VSMC apoptosis significantly reduced intimal thickening in a porcine vein graft model (George et al, 2000). Adenoviral delivery of wild type p53 which promotes VSMC apoptosis has also been studied in human saphenous vein in vitro studies (George et al, 2001). Induction of VSMC apoptosis by overexpression of p53, without a detectable reduction in VSMC proliferation, led to a significant reduction, >70%, in intimal thickening (George et al, 2001). Studies using a porcine arterio-venous bypass model are currently been underway to determine if this cytostatic strategy reduces intimal thickening in vivo. Despite initial concerns, this pro-apoptotic strategy with TIMP-3 and p53 did not lead to a

loss of VSMC density or thinning of the graft wall that may lead to aneurysm (George et al, 2000, 2001).

E. Anti-migration/matrix remodelling

Cell migration is critical to intimal thickening and requires remodelling of the matrix by proteolytic enzymes such as matrix-degrading metalloproteinases (MMPs) and plasmin. The tissue inhibitors of matrix-degrading metalloproteinases (TIMPs) regulate the proteolytic activity of MMPs whilst the balance of plasminogen activators and plasminogen activator inhibitor-1 (PAI-1) regulate plasmin. Increased MMP activity has been demonstrated both in vitro (George et al, 1997) and in vivo (Southgate et al, 1999) models of vein graft failure. Local overexpression of TIMPs (1, 2 and 3) reduced intimal thickening in a human in vitro model of vein graft failure (George et al, 1998a,b, 2000). Furthermore, ex-vivo delivery of TIMP-3 gene reduced MMP activity and intimal thickening in a porcine vein graft model (George et al, 2000), (**Figure 2**). Using the recently established mouse model of vein grafting the potential of gene therapy of TIMPs was further illustrated (Hu et al, 2001). Inhibition of plasminogen activators also inhibits intimal thickening in a human in vitro model of vein graft failure (Quax et al, 1997). Intimal thickening after balloon injury of the rat carotid was reduced by 35% at 4 weeks after adenoviral delivery of a hybrid protein which consists of the amino-terminal fragment of urokinase plasminogen activator linked to bovine pancreas trypsin inhibitor, a potent inhibitor of plasmin (Lamfers et al, 2001). Gene transfer of TIMPs has not been used yet in adversing cerebral ischemia (Napoli, 2002).

F. Anti-ischemia/reperfusion, oxidative stress, inflammation

Molecular therapies targeted at scavenging the excess of reactive oxygen species generated locally or protecting resident cells from their downstream effects may be useful in the prevention of vein graft failure. Gene therapy using naturally occurring cytoprotective and anti-oxidant mechanisms including heat shock protein-70 (Jayakumar et al, 2000), and scavenging enzymes such as catalase (Danel et al, 1998), superoxide dismutase (Li et al, 2001), and heme oxygenase-1 (Yang et al, 1999) have proven efficacy in models of arterial and lung injury and cardiac reperfusion but to date have not been used in vein grafts. Similarly, gene transfer of TIMPs has not been used in cerebral ischemia (Napoli, 2002). Although pre-treating the vein with anti-oxidant gene therapy is an attractive strategy it may be difficult in practice because of the immediate onset of reperfusion after implantation and the time delay before adequate transgene expression. However, antioxidant gene therapy might be advantageous for later stages of graft healing, as oxidative stress is a consequence of inflammation (West et al, 2001). Possible anti-inflammatory strategies include overexpression of nitric oxide synthase (NOS), soluble adhesion molecules and CC-chemokine blockade. By far the most progress has been made with NOS overexpression, probably since it also inhibits thrombosis formation and VSMC proliferation (Cable et al, 1997). Ex vivo gene transfer of endothelial (e)NOS to canine ipsilateral femoral vein grafts (Matsumoto et al, 1998) and inducible (i)NOS to porcine jugular (Kibbe et al, 2001) and intraoperative gene transfer of neuronal (n)NOS to jugular vein grafts in rabbits (West 2001) significantly reduced (30% to 50%) intimal thickening. However, only in the latter study was a reduction in inflammation observed. A current clinical trial (Cardion, Inc, Cambridge, Mass) is examining the effects of liposome-mediated iNOS gene transfer to coronary arteries after angioplasty for the prevention of restensosis but no such trials are currently examining the potential for prevention of vein graft failure. Despite demonstration of the ability to overexpress a soluble form of the vascular adhesion molecule in vein grafts and highlighting the potential for reducing vein graft failure (Chen et al, 1994), its efficacy has not been demonstrated. Furthermore, the ability of overexpression of 35K, a CC-chemokine inactivator, to inhibit inflammation has only been demonstrated in the peritoneum of mice (Bursill et al, 2003).

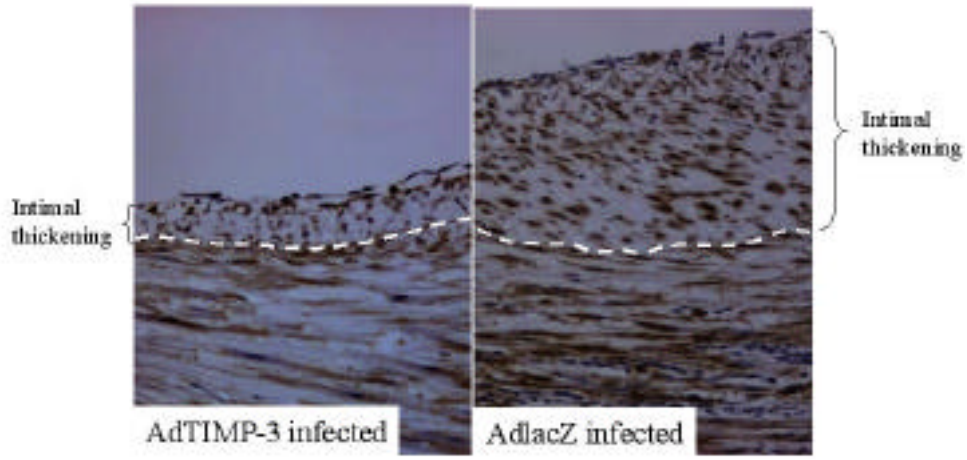


Figure 2: Adenoviral-mediated gene transfer of TIMP-3 reduced intimal thickening in vein grafts. Transverse sections stained for α-smooth muscle cell actin illustrate that intimal thickening was dramatically reduced in porcine arterio-venous vein grafts at one month by Ad-mediated over-expression of TIMP-3 compared to controls (AdlacZ). White dotted line indicates the intimal/medial boundary.

IV. Gene therapy and restenosis

Treatment of symptomatic coronary artery atherosclerotic plaques by angioplasty leads to vascular responses including intimal thickening and constrictive remodelling causing restenosis in approximately 30% of initially successfully treated patients. Although stents prevent constrictive vascular remodelling, they induce vascular injury eventually leading to intimal thickening and thereby restenosis. Gene therapy has been perceived as attractive to treat restenosis as it can be delivered locally and appears to be able to treat excessive vascular cell proliferation.

To date, a number of small (rat, mice) or large size animal modes (rabbit, pig) have been used to evaluate the potential of many gene therapy approaches for restenosis. The gene therapy strategies for treatment of restenosis are summarized below and also in **Figure 1**. However, despite the successful use of gene therapy to treat animal restenosis by various approaches, application of gene therapy to prevent restenosis in man has only been carried out using a re-endothelialization strategy with VEGF. Before further clinical trials are initiated a better understanding of vascular biology, gene expression, vector design, and catheter-tissue interactions is required. It must also be mentioned that the efficacy of sirolimus (rapamycin) for the treatment of in-stent restenosis (Serruys et al, 2002; Sousa et al, 2003) has reduced the impetus for designing gene therapy for in-stent restenosis.

A. Biological processes involved in restenosis and molecular targets in restenosis

The two major components that lead to restenosis are intimal thickening and negative (constrictive) remodelling. Intimal thickening following experimental injury involves a combination of many processes, including VSMC and adventitial cell migration, proliferation, and matrix deposition. Negative remodelling, which only occurs after angioplasty and not after stent placement may also arise from many processes, including VSMC apoptosis, medial and adventitial fibrosis and matrix remodelling. However, restenosis, both in the absence and in the presence of stents, is primarily due to VSMC accumulation. Since mural thrombi may aggravate restenosis by contributing directly to cell proliferation, anti-thrombotic strategies have received attention. Finally, strategies that accelerate re-endothelialization of the injury artery have been investigated.

B. Inhibition of VSMC proliferation

Cytotoxic strategies have been tested based on the expression of enzymes capable of converting nucleoside analogues into toxic metabolites that impair DNA replication and consequently cause death of transduced cells entering S phase. Adenoviral delivery of thymidine kinase (tk), a gene from herpes simplex virus (HSV), followed by ganciclovir treatment led to death of tk-expressing cells and reduced intimal thickening after injury of rat and rabbit arteries (Guzman et al, 1994; Simari et al, 1996). Similarly, expression of cytosine deaminase in the presence of 5-fluorocytosine caused a 45% reduction of stenosis (Harrell et al, 1997). Endogenous inducers of cell death have also been utilized. Delivery of the tumour suppressor p53 to injured rat carotid arteries reduced intimal thickening (Yonemitsu et al, 1998), as did gene transfer of FasL (Luo et al, 1999). Some caution has been applied to the use of cytotoxic gene therapy for restenosis, since VSMC viability is essential for the integrity of the lesion, particularly the fibrous cap, and thereby the stability of atherosclerotic plaques. In addition, promotion of apoptosis in injured vessels may increase intimal thickening, since overexpression of fortilin, a recently characterised, negative regulator of apoptosis reduced intimal thickening in injured rat arteries (Tulis et al, 2003).

It has been well documented that cytostatic genetic strategies using antisense oligonucleotides (ODN), decoy ODN and gene transfer of cell cycle inhibitory genes (Li et al, 1999) limit VSMC proliferation and inhibit intimal thickening following experimental injury. Despite encouraging results using antisense ODN to immediate early genes such as c-myb (Simons et al, 1992) and c-myc (Shi et al, 1994) and promoters of cell cycle such as cyclin B and CDK-2 (Morishita et al, 1994), where intimal thickening was inhibited between 40 and 84% to in rat and in some cases also porcine injured arteries some years ago, this strategy appears to have made little progress recently. This is despite the observation that co-transfection of combinations of these antisense resulted in further inhibition (Morishita et al, 1994). Transfer of retinoblastoma protein (Rb) to restrict the cell cycle, into rat and porcine injured arteries prevented intimal thickening (Chang et al, 1995). Similarly, overexpression of the CDK inhibitors p21 and p27 resulted in reduction of intimal thickening both in rat and porcine injured arteries (Chang et al, 1995; Yang et al, 1996; Chen et al, 1997). Furthermore, overexpression of a mutated form of p21 was able to reduce restenosis in hypercholesterolemic mice by enhancing vascular apoptosis and reducing VSMC proliferation (Condorelli et al, 2001). A further strategy that has been examined is the inhibition of signalling molecules. H-ras, a key protein in signal transduction, mediates mitogenic signals, therefore blocking this early signal transduction. Application of an adenoviral dominant negative H-ras and Gβγ-binding peptide affected downstream signalling events and reduced intimal thickening by 70-80% (Ueno et al, 1997; Iaccarino et al, 1999). Targeting of transcription factors by gene therapy is also a strategy of interest. Inhibition of NFκB and E2F, cytoplasmic transcription factor using antisense ODNs in balloon-injured rat carotid arteries reduced intimal thickening by approximately 70% (Autieri et al, 1995; Morishita et al, 1995). Overexpression of the growth arrest homeobox gene (GAX) reduced intimal thickening by 50-70% in rat and rabbit injury models (Maillard et al, 1997; Smith et al, 1997). Although the use of transcription factors as targets for gene therapyapy in restenosis appeared promising, it should be noted that these transcription factors are also involved in several mechanisms regulating vascular wall homeostasis.

Control of VSMC proliferation has also been attempted by inhibition of growth factor expression and overexpression of inhibitory growth factors and cytokines. Delivery of basic fibroblast growth factor (bFGF) (Hanna et al, 1997) as well as platelet-derived growth factor-β (PDGF-β) (Deguchi et al, 1999) antisense ODN and TGF-β ribozyme ODN (Yamamoto et al, 2000) inhibited intimal thickening by 60-90% in injured rat carotid arteries. Similarly, adenoviral delivery of the extracellular region of the PDGF-β receptor and of endovascular PDGF-β receptor antisense ODN reduced intimal thickening in injured rat arteries (Sirois et al, 1997; Noiseux et al, 2000). Activin, a TGF-β-like factor that induces a contractile phenotype in VSMCs, reduced intimal thickening by more that 70% in injured mouse femoral arteries (Engelse et al, 2002). The inhibitory cytokine interferon-g delivery by Ad-mediated gene therapy reduced intimal thickening in a porcine model of arterial injury (Stephan et al, 1997).

C. Cell migration and matrix remodelling

Constrictive (negative) remodelling plays a very important in human restenosis particularly in the absence of a stent (Mintz et al, 1996), therefore gene therapy strategies aimed at reducing intimal thickening alone are unlikely to be successful in humans following angioplasty. Post injury intimal thickening is also reliant on VSMC migration, which requires remodelling of the extracellular matrix that surrounds the VSMC. Adenoviral gene transfer of tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2 reduced intimal thickening (Cheng et al, 1998; Furman et al, 2002). A combination of anti-proliferative and anti-migratory approaches may therefore be useful.

D. Anti-thrombotic strategy

A number of studies have focused on seeding stents with genetically modified endothelial cells with increased fibrinolytic of anticoagulant activity (Dichek et al, 1989, 1996; Dunn et al, 1996). Although seeding stented vessels with endothelial cells overexpressing tPA and uPA produced anti-thrombotic activity (Dichek et al, 1996), overexpression of tPA was associated with increased detachment of seeded cells (Dunn et al, 1996).

Another strategy to prevent thrombosis as well as intimal thickening is to inhibit platelet activation or aggregation or to increase nitric oxide (NO). NO is vasoprotective by inhibiting platelet and leukocyte adhesion, inhibiting VSMC proliferation and migration and promoting endothelial cell survival and proliferation (Li et al, 1999); therefore, nitric oxide synthase (NOS) that increases NO production was proposed as a suitable candidate to treat restenosis. Delivery of endothelial (e)NOS by non-viral methods (von der Leyen et al, 1995) and adenoviruses (Chen et al, 1998; Janssen et al, 1998; Varenne et al, 1998) reduced intimal thickening by 37-70% in rat and pig injured arteries. Interestingly, adenoviral delivery of inducible (i)NOS by adenoviruses to rat injured arteries almost completely (95%) inhibited intimal thickening, whilst reduced it by only 50% in porcine injured arteries (Shears et al, 1998), illustrating that the degree of response differs greatly between different animal models. Furthermore, administration of the iNOS Ad could not mediate regression of established intimal thickening.

D. Re-endothelialization

As regeneration of the endothelium is associated with reduction in thrombotic and proliferative processes in the vessel wall it has been seen as a potential strategy of gene therapy for restenosis. Local intravascular and extravascular expression of vascular endothelial growth factor (VEGF), a potent endothelium specific angiogenic factor, using plasmid DNA accelerated re-endothelialization and decreased intimal thickening after arterial injury in rabbit models (Asahara et al, 1996; Laitinen et al, 2000), and reduced in-stent restenosis by 50% (Van Belle et al, 1997).

The feasibility of this approach was tested in a small clinical trial, in which VEGF plasmid/liposome gene transfer after angioplasty was seen to be safe and well tolerated (Laitinen et al, 2000). A recently published larger clinical trial was designed to test the feasibility, tolerability and efficacy of VEGF gene therapy to prevent restenosis after stenting (Hedman et al, 2003). The overall restenosis rate in this study was surprisingly low (6%), virtually precluding the detection of a difference among treatments. Nevertheless, the results establish feasibility and provide safety data on the used of naked DNA and Ad to express VEGF. This strategy is perceived attractive as it is trying to mimic nature's inhibitory strategy to limit intimal thickening, but we await clinical evidence of its success. The use of VEGF is also attractive as it should be endothelial cell specific; however, there are safety concerns in respect to tumour growth as VEGF is involved in induction and progression (Huang et al, 2003).

V. Gene therapy for hypertension

Gene therapy for essential hypertension represents is an enormous challenge due to the complex polygenic trait that underlies human essential hypertension. Gene therapy is however attractive since it offers the opportunity to treat the disease with a single administration rather than daily drug regimens. Essential hypertension is associated with endothelial dysfunction and contributes significantly to cardiovascular risk. Gene therapy would, therefore, target specific systems with the explicit aim of lowering blood pressure and reducing end organ damage. Unlike other disease targets discussed above, gene therapy for hypertension requires the use of strategies to provide long-term effects on blood pressure. These have included antisense/ribozyme strategies to block systems that regulate blood pressure as well as vasodilator strategies using overexpression of pro-vasodilator genes.

Preclinical studies on gene therapy for hypertension have taken two main approaches (Phillips, 2002). First, extensive studies on gene transfer to increase vasodilator proteins (kallikrein, atrial natriuretic peptide, adrenomedullin, and endothelin nitric oxide synthase)

have been carried out in different rat models (Lin et al, 1995; Chao et al, 1996, 1997; Lin et al, 1997; Chao et al, 1998 a, b; Yayama et al, 1998; Alexander et al, 1999; Dobrzynski et al, 1999; Lin et al, 1999; Alexander et al, 2000; Dobrzynski et al, 2000; Wolf et al, 2000; Zhang et al, 2000; Wang et al, 2001; Emanueli et al, 2002). Using these approaches, blood pressure can be lowered for 3-12 weeks with the expression of these genes. Second, an antisense approach, which began by targeting angiotensinogen and the angiotensin type 1 (AT1) receptor, has now been tested independently by several different groups in multiple models of hypertension (Katovich et al, 1999; Tang et al, 1999; Wang et al, 2000; Kimura et al, 2001). Other genes targeted include the β1-adrenoreceptor, TRH, angiotensin gene activating elements, carboxypeptidase Y, c-fos, and CYP4A1 (Gardon et al, 2000; Phillips, 2001; Tomita et al, 2002). There have been two methods of delivery antisense, short ODNs, and full-length DNA in viral vectors. All the studies show a decrease in blood pressure lasting several days to weeks or months. ODNs are safe and particular non-toxic. The decreased hypertension after systemic adeno-associated virus delivery antisense to AT1 receptors in adult rodents for up to 6 months, may constitute a good incentive for testing the antisense ODNs first and later the AAV (Kimura et al, 2001; Phillips 2001).

Hypertension is also the presenting feature of some of these disorders, such as congenital adrenal diseases, and adrenal and pituitary tumors. Preclinical data indicate that gene transfer to both the adrenal gland and the pituitary is not only feasible but also quite efficient (Alesci et al, 2002).

A. Inhibition of vasoconstrictor genes

This has been achieved using antisense oligonucleotides to block the renin-angiotensin system. For example, Wielbo et al (1996) used DNA/liposomes complexes containing angiotensinogen antisense and lowered mean arterial pressure, angiotensinogen and angiotensin II levels in adult spontaneously hypertensive rats following systemic administration. These highly effective results are somewhat surprising when it is realised that the in vivo uptake of DNA/liposome complexes into the vasculature and organs is very poor when delivery intravenously. Not surprisingly viral vector systems have also been engineered to deliver antisense. Using a retroviral system to deliver antisense against the angiotensin type-1 receptor to young (5 day old) hypertensive and normotensive animals, blood pressure was significantly lowered selectively in the hypertensive animals (Lu et al, 1996). Interestingly, the effect of the antisense was sustained for 90 days while losartan had the expected transient effect of less than 24 hours. This does highlight the clinical relevance of such technology to provide sustained benefit compared to traditional pharmacological regimens. However, in the light of recent clinical experience using retroviral vectors with development of leukaemia on phase I trial (Cavazzana et al, 2000), the use of retroviral vectors is unlikely to be developed in this disease. Other studies have also highlighted the benefit of viral delivery of antisense (Wang C et al, 1995; Martens et al, 1998; Reaves et al, 1999; Tang et al, 1999; Wang H et al, 1999).

B. Vasodilator overexpression

There are a number of candidate genes for overexpression that may provide therapeutic benefit of different aspects of hypertension. These include kallikrein, adrenomedullin, nitric oxide synthase and superoxide dismutase. Kallikrein cleaves kininogen producing kinin peptide, which in turn stimulates the release of the vasodilators prostacyclin, endothelium-derived hyperpolarising factor and nitric oxide. Based on this principle, infusion of naked DNA expressing kallikrein reduced blood pressure for 6 weeks (Wang et al, 1995). Comparative studies showed that naked DNA plasmids and adenoviral vectors both proved effective (Chao et al, 1997). Kallikrein delivery using viruses has also been established as an anti-hypertensive strategy in different models demonstrating the potential benefit of this strategy and the potency of the transgene (Dobrzynski et al, 1999; Wolf et al, 2000).

Adrenomedullin also causes vasodilation. Adenoviral-mediated overexpression of adrenomedullin in hypertensive rats led to a blood pressure drop of 41 mm Hg 9 days after tail vein injection (Dobrzynski et al, 2000). This lasted nearly 20 days. Again, proof of this strategy was realised when other studies gained similar findings in different labs and models of hypertension (Zhang et al, 2000; Wang et al, 2001).

Targeting endothelial dysfunction is highly attractive for gene therapyapy. Endothelial dysfunction is characterised by reduced nitric oxide (NO)-mediated vasodilation and a reduction in available NO. The loss of NO leads to deleterious effects on platelet aggregation and adhesion, smooth muscle proliferation, inflammation and increased oxidative stress in the vessel wall. Improving the bioavailability of NO, therefore, is a highly logical strategy to improve a number of key processes that are integral to vessel wall homeostasis in order to reduce blood pressure. This can be achieved by increasing NO production itself through nitric oxide synthase (NOS) gene delivery or by preventing NO degradation by superoxide dismutase (SOD) gene transfer. A number of studies have addressed these issues. An early study established such a concept by systemic delivery of naked DNA encoding the endothelial form of NOS (eNOS) with a significant reduction in blood pressure that lasted for at least 12 weeks (Lin et al, 1997). Again, such effects with naked DNA are astonishing since little uptake was achieved in vivo and the majority was sequestered to the liver. It is important to note that targeting gene delivery to the endothelium is extremely difficult using currently available vector systems when the delivery mode is intravenously. The liver sequesters the vast majority of all commonly used vector systems with relatively little uptake by the endothelium itself. This has restricted studies to local applications of gene delivery to selected blood vessels in vivo. Adenoviral delivery of eNOS or SOD3,

but not SOD-1 or –2 are able to improve endothelial function in carotid arteries in the spontaneously hypertensive stroke-prone (SHRSP) rats (Alexander et al, 1999, 2000; Fennell et al, 2002).

VI. Therapeutic angiogenesis

Therapeutic angiogenesis represents a novel strategy for the treatment of vascular insufficiency. It is based on supplementation with angiogenic growth factors to enhance native angiogenesis in critical myocardial or peripheral ischaemia. Angiogenic growth factors have been delivered both as protein and by way of gene transfer and have demonstrated positive results (Yla-Herttuala et al, 2003). The recent insights in the molecular basis of angiogenesis have resulted in great interest in the gene therapy field. However, because of the rapid evolution and enthusiasm in the field, angiogenic molecules have been tested without a complete understanding of their mechanism of action. Among the angiogenic growth factors used in pre-clinical studies, VEGF165 and VEGF121, FGF1, FGF2 and hepatocyte growth factor (HGF) have all shown significant improvement of native angiogenic response to ischemia, resulting in accelerated rate of perfusion, (see reviews by Hammond et al, 2001) (Emanueli et al, 2001; Manninen et al, 2002). Besides growth factors a number of other substances have been investigated, such as human tissue kallikrein (Emanueli et al, 2001), angiopoietin (Shyu et al, 1998), leptin (Bouloumie et al, 1998) and thrombopoietin (Brizi et al, 1999).

Although difficulties have been encountered in the field of gene therapy, great progress has been made in the field of pro-angiogenic gene therapy. It has been suggested that this is because the long-term gene expression is not required for therapeutic vascular growth and the current gene therapy vectors induce at least some physiological improvement (Yla-Herttuala et al, 2003). Over 23 clinical trials have been initiated; approximately half are for peripheral disease and the other half for coronary heart disease. The first set of clinical trials involved pioneering attempts to overexpress VEGF165 with naked DNA (Isner et al, 1996; Baumgartner et al, 1998; Losordo et al, 1998) and adenoviruses (Rosengart et al, 1999). The second phase of trials were small, uncontrolled trials using naked DNA and adenoviruses to overexpress VEGF165 and VEGF121; many of these had positive results (Symes et al, 1999; Laitinen et al, 2000; Rajagopalan et al, 2001). Only recently, the third set of clinical trials has begun to test the potential of this gene therapy fully. These randomised, controlled and blinded trials have involved larger numbers of patients and defined primary and secondary endpoints (Grines et al, 2002; Makinen et al, 2002; Stewart et al, 2002; Hedman et al, 2003; Rajagopalan et al, 2003). Several of these have been judged positive according to primary and secondary endpoints but it has been suggested that this may not be transferable to a clear-cut clinical benefit (Yla-Herttuala et al, 2003).

Critically ischaemic lower limbs from diabetes that are not suitable candidates for surgical endovascular approaches may be amenable to gene therapy for therapeutic angiogenesis. Diabetes impairs endogenous neovascularization of ischaemic tissues due to a reduced expression of VEGF (Rivard et al, 1999) and HGF (Taniyama et al, 2001). Consequently Ad-mediated overexpression of VEGF and plasmid HGF restored neovascularization in mouse and rat models of diabetes, respectively (Rivard et al, 1999; Taniyama et al, 2001). Enhanced angiogenesis by such strategies also improves neuropathy both when growth factors including VEGF, are given alone (Rissanen et al, 2001) or in conjunction with the prostacyclin synthase gene (Koike et al, 2003). Furthermore, a small clinical trial which included 6 diabetic patients with critical leg ischaemia, observed neurologic improvement and therapeutic angiogenesis after plasmid injections of VEGF165 in the muscles of the ischaemic limb (Simovic et al, 2001). Inhibition of angiogenesis may also have therapeutic potential for the treatment of retinopathy, since lentiviral delivery of angiostatin inhibited neovascularization in a murine proliferative retinopathy model (Igarashi et al, 2003).

Although, this strategy has made great progress in the last decade there are still some unresolved issues. For example is administration of a single angiogenic molecule sufficient? Will administration of VEGF lead to toxic effects such as oedema? Will an angiogenic factor be suitable for myocardial and peripheral angiogenesis? Since the same adenoviral VEGF121 gave positive effects in the myocardium (Stewart et al, 2002) but failed in peripheral vascular disease (Rajagopalan et al, 2003), will VEGF be proven clinically benefial? Some caution has been cast on the potential of VEGF gene therapy by the observation that VEGF enhances atherosclerotic plaque progression in both mice and rabbits (Celletti et al, 2001). Are other VEGF homologues safer options? Increased lymphogenesis and reduced oedema is observed with VEGFC and VEGFD (Yla-Herttuala et al, 2003).

VII. Future directions

Recent advances through preclinical studies have raised the profile of gene therapy in some vascular diseases, particularly with respect to angiogenic gene therapy in the myocardium and peripheral vasculature as well as in vein graft disease. These studies, presently in phase II, highlight the potential of the technology for relieving symptoms of human vascular diseases.

Despite the lack of dramatic cures, a decade of clinical trials has provided important news about the strengths and weaknesses of current vectors. Both adenoviruses and liposomal vectors have been shown to be able to transduce transgenes in patients with a variety of disorders. From this work, it is now extremely clear that the expression is temporary and is associated with an inflammatory response. However, there are some important points to consider. First, with respect to myocardial and peripheral vascular gene transfer clinical trials, these have been performed with single pro-angiogenic genes with gene delivery using sub-optimal vector systems (e.g. naked DNA/adenoviral vectors). With

respect to the former, angiogenic gene therapy may be significantly more therapeutic with respect to collateral vessel formation with a combination of therapeutic genes rather than single gene therapy strategies. With recent advances in adenoviral vector technology [e.g. using "gutted" adenoviral vectors (Kochanek et al, 1996; Parks et al, 1996)] the cloning capacity required for such studies is now available. Equally, the gutted adenoviral vector systems are less immunogenic in vivo and would allow longer term overexpression of transgenes that in turn may promote sustained angiogenic effects. It is known that vascular cell uptake by these vectors (all based on serotype 5 adenoviruses) is extremely poor in comparison to other cells, such as hepatocytes in the liver (Nicklin et al, 2001). Indeed, pre-clinical experiments have shown that local delivery of adenoviruses serotype 5 vectors to the vasculature leads to virion dissemination, not only to the liver but also to testes and other organs posing additional safety concerns (Hiltunen et al, 2000; Baker, 2002).

Given the limited ability of liposomes and adenoviruses to enable long-term gene expression, and given the poor in vivo performance of retroviruses, the AAV vectors are being developed. This virus is smaller than the adenovirus and has a relatively low-capacity size. However, it allows for long-term gene expression (ie, months to years) with only minimal induction of inflammation or antiviral immune responses. A better understanding of the life cycle of this virus, along with improved production techniques, has allowed investigators to conduct clinical trials with AAV in diseases such as hemophilia and cystic fibrosis (see http://www.wiley.co.uk/wileychi/genmed/clinical/). Preclinical data in mice injected intramuscularly with an AAV-human alpha-1-antitrypsin (1AT) vector are encouraging (Xiao et al, 1998; Phillips et al, 2002).

To date, the major problem in gene therapy remains the relative inefficiency of current vectors. Currently, this inefficiency, coupled with a relatively poor specificity of most vectors, requires the delivery of large doses of vector. This is both expensive and more likely to lead to side effects. Pathophysiological questions still remain about which and how many cells need to be transduced to obtain a clinical response. One new and very exciting area of gene therapy that has not yet reached clinical trials is the "gene correction" (Gamper et al, 2000; Metz et al, 2002). It is possible to design oligonucleotides that bind to areas of single-nucleotide changes that are associated with abnormal functions and to catalyze corrections of the nucleotide errors. This concept clearly has been demonstrated to work in cell cultures and in animal models, although the efficiency is still quite low. With the development of better oligonucleotides and improved delivery methods, this approach will likely be tested first in diseases such as hemophilia and 1AT.

When it is considered that angiogenic gene therapy should be highly localised due to potential side effects [including potentiation of atherosclerosis (Celletti et al, 2001) and development of cancer (Lee et al, 2000)] other vector systems should now be considered. The choice of potential new vectors is broad and must be considered with caution and evaluated based on current knowledge of existing systems (de Nigris et al, 2003). Additional evidence now suggests that the vast majority of AAV genomes remain in a non-integrative capacity within infected cells (Nakai et al, 2001; Schnepp et al, 2003) further supporting the safety of this vector system. Of equal potential are adenoviral vectors originating from different serotypes. Previous pre-clinical data support of the notion that novel vector systems can be isolated for the capacity to efficiently infect an individual tissue type (Havenga et al, 2001, 2002). For example, adenoviruses based on serotype 16 have a high propensity to transduce both endothelial cells and smooth muscle cells than serotype 5 vectors (Havenga et al, 2001). Again, like AAV-2, this may provide a system through which to optimise gene delivery for defined gene therapeutic applications. The use of cell selective promoters (tissue-specific expression) to drive transgene expression will add a further level of selectivity to such systems. The combined use of vectors and immuno-suppressors may be also reasonable.

Gene therapy remains the key link between advances in genetics and genomics and the translation of this knowledge into useful outcomes for patients. Although progress has been slower than hoped for, clear advances are being made; gene therapy will probably find a number of key therapeutic niches. Together, these modifications will enhance the utility and safety of gene therapy as transition from pre-clinical to clinical gene therapy proceeds for the vascular system and its diseases.

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