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REVIEW

Cardiovascular gene therapy: current status and therapeutic potential

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Gene therapy is emerging as a potential treatment option in patients suffering from a wide spectrum of cardiovascular diseases including coronary artery disease, peripheral vascular disease, vein graft failure and in-stent restenosis. Thus far preclinical studies have shown promise for a wide variety of genes, in particular the delivery of genes encoding growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) to treat ischaemic vascular disease both peripherally and in coronary artery disease. VEGF as well as other genes such as TIMPs have been used to target the development of neointimal hyperplasia to successfully prevent vein graft failure and in-stent restenosis in animal models. Subsequent phase I trials to examine safety of these therapies have been successful with low levels of serious adverse effects, and albeit in the absence of a placebo group some suggestion of efficacy. Phase 2 studies, which have incorporated a placebo group, have not confirmed this early promise of efficacy. In the next generation of clinical gene therapy trials for cardiovascular disease, many parameters will need to be adjusted in the search for an effective therapy, including the identification of a suitable vector, appropriate gene or genes and an effective vector delivery system for a specific disease target. Here we review the current status of cardiovascular gene therapy and the potential for this approach to become a viable treatment option.
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Introduction

Cardiovascular disease has been estimated to cause 17 million deaths per year globally and is one of the most common causes of death in the developed world (WHO, 2006). An epidemic of cardiovascular disease is now taking place and it has been estimated that it will be the leading cause of mortality in the developing world within the next decade (WHO, 2006). The emerging field of gene therapy is recognised as having the potential to add to the therapeutic armamentarium for cardiovascular disease by treating patients with disease, which is resistant to current approaches (Isner, 2002).

The introduction of transgenes into the vasculature has the advantage of providing long-term expression where required and also allows the therapy to specifically target the disease process involved. Ease of access to the circulatory system is another advantage for this type of therapy. Gene therapy in addition to helping control symptomatic presentation of cardiovascular disease may also be able to reverse the pathological processes involved. However, to achieve these objectives three goals must be met: suitable vectors must be generated, a suitable gene or group of genes must be identified and an appropriate delivery system needs to be developed (Figure 1). Optimal characteristics of these features may vary depending on the disease being targeted.

This review will focus on four potential therapeutic targets for cardiovascular gene therapy using two therapeutic approaches. The first approach uses therapeutic angiogenesis to treat peripheral vascular disease and ischaemic heart disease. The second approach attempts to use gene therapy to prevent neointimal hyperplasia, which targets vein graft bypass failure and in-stent restenosis. Pharmacotherapy often requires long-term administration while rarely addressing the underlying pathophysiology. Interventional techniques are subject to failure in a significant proportion of cases. The formation of neointimal hyperplasia in vein grafts or stented vessels may cause failure of these therapeutic approaches. Even with optimal pharmacotherapy some forms of cardiovascular disease may be refractory to treatment and prognosis remains poor for many patients.

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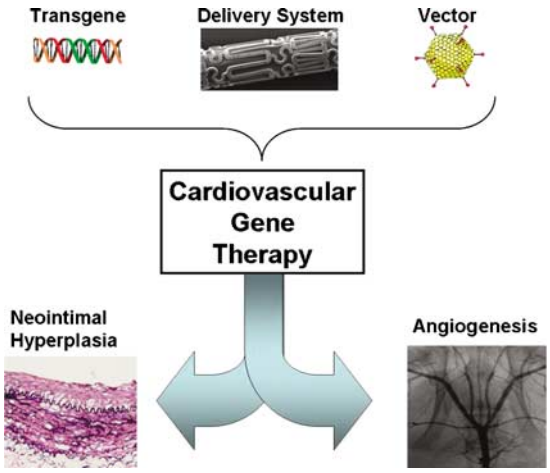

Figure 1 The factors required for successful cardiovascular gene therapy.

Vectors

Choosing the correct vector for gene therapy applications is probably the biggest challenge faced by gene therapists (Baker, 2004). The ideal vector would be noncytotoxic, highly efficient, have a large capacity, possess an appropriate tissue tropism preventing distal spread and be non-immunogenic. Recombinant viruses represent an efficient system for delivering genes to human tissues. Viruses commonly employed include retroviruses such as lentivirus, adenovirus and adeno-associated virus (AAV). However, there are many hazards associated with using viral gene therapy in humans. These include the induction of a host immune response, random insertional mutagenesis, the presence of wild-type vector in the administered preparation and unsuitable tissue tropism.

Viral vectors

Retroviruses were among the first vectors used for delivery of specific transgenes to the vasculature (Nabel *et al*., 1990). Retroviruses are RNA viruses, which insert their viral genome into the host chromosome. Although this results in prolonged tissue expression, it opens the putative possibility of random insertional mutagenesis and other forms of carcinogenesis. Despite this, retroviral vectors have been the most widely used vector in gene therapy clinical trials to date. The use of this vector to treat X-linked severe combined immunodeficiency syndrome, resulted in three patients developing a T-cell proliferation (Hacein-Bey-Abina *et al*., 2003) probably due to an altered IL-2 receptor with subsequent development of lymphoma (Woods *et al*., 2006). Moreover, retroviral vectors have poor uptake into nonmitotic cells, which are commonly found in vascular tissues. Developments to improve the safety and efficacy of these vectors include pseudotyping of retroviruses which changes the tropism of a virus due to alterations in its capsid proteins (Burns *et al*., 1993).

Lentiviruses are a subgroup of retroviruses which unlike the other members of the group are capable of transducing nondividing cells such as those found in the blood vessel wall (Totsugawa *et al*., 2002). The low titre achievable and safety concerns regarding random insertional events are drawbacks of this vector for use in gene therapy. However, initial work by Dishart *et al*. (2003) suggests that lentiviruses pseudotyped with the vesicular stomatis virus glycoprotein efficiently transduce vascular cells *in vitro*. Similarly, an *in vivo* gene therapy strategy using such vectors to deliver vascular endothelial growth factor (VEGF) and angiopoietin 2 to hindlimb ischaemia in a rabbit model has been effective (Conklin *et al*., 2005). Although to date, lentivirus remains little used in vascular gene therapy, its lower immunogenicity may offer advantages over other vector types.

Adenoviruses are double-stranded DNA viruses and unlike retroviruses do not integrate into the host genome and therefore do not have the associated risk of insertional mutagenesis. Adenoviral vectors are becoming increasingly popular as vectors in gene therapy with high titres achievable and a broad tissue tropism (OhNo *et al*., 1994; Varenne *et al*., 1998). They have been shown to be particularly effective in vascular cell types and can transduce quiescent cells efficiently. However, they may result in the induction of an immune response, which can decrease their effectiveness. The advent of the so-called 'gutless' or helper dependent adenoviral vectors lacking any viral genetic elements may improve longer-term delivery by reducing the host immune response. They also have larger capacity than earlier generation adenoviruses and may allow multiple gene delivery in a single vector (Jozkowicz and Dulak, 2005).

AAVs are nonpathogenic parvoviruses that require cells to be doubly infected by a helper virus, such as adenovirus, to replicate in nature. They have recently emerged as potential gene therapy vectors. They have a small genome (<5 kb) and carry single-stranded DNA. Like adenoviral vectors, they can infect nondividing cells and can be concentrated to relatively high titres. In contrast to adenoviruses and similar to lentiviruses they can integrate into the host genome in nature but this property is lost in recombinant AAV, eliminating the risk of insertional mutagenesis (Young *et al*., 2000). AAVs have lower immunogenic potential than adenoviral vectors coupled with a prolonged expression. Although the most widely used serotype, AAV2, initially demonstrated some promise in the vasculature (Nicklin *et al*., 2001), further *in vivo* studies demonstrated AAV1 and 5 to be superior *in vivo* (Chen *et al*., 2005), whereas AAV7 and -8 have been shown to transduce endothelial cells poorly (Denby *et al*., 2005). The various serotypes identified have differing tissue tropisms, which are currently the source of active investigation. These serotypes may, in addition, be enhanced through tissue tropism modifications to their capsid proteins. Using *in vivo* biopanning, a study by Work *et al*. (2006) significantly improved delivery of AAV2-modified vectors to the vasculature.

Nonviral vectors

Naked plasmid DNA has been used to deliver genes effectively to the vasculature and although the efficiency is low this limitation can be overcome by the use of a potent secretory transgene such as VEGF (Majesky, 1996). Attempts

to use artificially created liposome's to carry naked DNA as a method of gene delivery to the vasculature initially met with low efficiency and transient gene expression (Laitinen *et al*., 1997), although this is improving and has shown potential in therapeutic applications (Hedman *et al*., 2003; Deiner *et al*., 2006). Various polymers have also been proposed for use to improve nonviral delivery of genes. Nonviral delivery of DNA may be augmented at various levels including systemic delivery using polysine derivatives or at the organ level using hydrogels (Putnam, 2006). The biophysical properties of these compounds may enhance the stability, concentration as well as uptake of DNA by cells (Putnam, 2006) and require a systematic examination of their properties in this regard.

Other physical methods of delivering genes such as electroporation useful in many organs such as skeletal muscle and liver may also have a role in ensuring uptake of transgenes in the vasculature after either adventitial or luminal delivery (Dean, 2003). Direct injection followed by electroporation of the heart may also improve plasmid or liposome-based gene delivery (Harrison *et al*., 1998; Wang *et al*., 2001). A secretory product may prove useful in providing a paracrine effect from the genetically modified cells.

Delivery systems

Local gene delivery to focal lesions in the coronary or peripheral vasculature is ideally suited to the use of catheter-based vector delivery. Percutaneous delivery of gene vector-containing solutions to the vasculature has been attempted using various balloon catheters in animal models and in human trials (Hedman *et al*., 2003; Sharif *et al*., 2004). Double-balloon catheters were the first used and these form a space between them that allows infusion of vector-containing solutions (Goldman *et al*., 1987; Rome *et al*., 1994). Although prolonged, efficient delivery of adenoviral vectors to the arterial wall has been demonstrated using this technique it is possible that the total occlusion of vessels for significant periods of time may cause problems due to downstream ischaemia. Other catheters have also been used which have porous balloons through which vector-containing solutions infuse under high pressure (Hedman *et al*., 2003). However, these catheters are associated with low efficiency of delivery in animal models (Wolinsky and Thung, 1990). Other variations of catheter type which have been used for gene therapy applications include hydrogel catheters where the vector is incorporated into a gel on the surface of the catheter and dispatch catheters which maintain vascular flow during vector delivery but require a long dispatch time of 60 min and summarised by Sharif *et al*. (2004). In contrast to the other catheters mentioned thus far, infiltrator catheters allow injection of vector into the vessel wall via micro injection needles which decreases the chances of systemic spread of the vector and may improve medial delivery of the transgene (Wang *et al*., 2003).

Stents are an ideal platform for localised delivery of vectors to the vascular wall due to their widespread use, safety and permanent scaffold structure. Currently available drug eluting stents have already demonstrated the ability of a stent to act as a reservoir for therapeutic agents. Gene delivery from stents has been achieved using cellular-based techniques (Koren *et al*., 2006) as well as various formulations on the stent surface to alter the vector binding/release kinetics such as polyurethane (Takahashi *et al*., 2003), phosphorylcholine (Wu *et al*., 2003; Sharif *et al*., 2006), polylactic-polyglycolic acid (Klugherz *et al*., 2000) and bisphosphonate (Fishbein *et al*., 2006). Alternatively specific antibody tethering may also be used to attach the vector to the stent and this has been shown using anti-adenoviral antibodies (Klugherz *et al*., 2002). Localised delivery of adenoviral/plasmid vectors to the vasculature may be confined to the intimal or neointimal layers due to anatomical barriers (Rome *et al*., 1994). However this may be sufficient because overexpression of angiogenic factors or inhibitors of neointimal proliferation in the endothelial layer are only transiently required for the applications discussed in this review. However, medial delivery can also be possible through an intact elastic lamina using other vector types or may also be achieved incidentally by rupturing of the elastic lamina due to angioplasty.

Gene therapy strategies

Therapeutic angiogenesis

Introduction. A significant proportion of patients with ischaemic vascular disease remain refractory to pharmacological therapies and are unsuitable candidates for percutaneous or surgical interventions. This has led to the development of therapies to stimulate neovascularisation, a strategy known as therapeutic angiogenesis. This approach attempts to stimulate the development of collateral blood vessels, which result in the formation of bypass vessels around occluded arteries. Studies by Folkman *et al*. (1971) suggested that the process of vasculogenesis (*de novo* vessel formation) and angiogenesis (branches budding from existing vessels) are both essential for the establishment and maintenance of a vascular supply, and that angiogenic growth factors are required for neovascularisation (Folkman *et al*., 1971). Angiogenesis occurs in response to stimuli including hypoxia, ischaemia, mechanical stretch and inflammation (Folkman and Shing, 1992) and is mediated by the proliferation and migration of preexisting, fully differentiated endothelial cells, which line the parent vessel walls.

A thorough understanding of the biology of angiogenesis is required to predict the most appropriate genes for use in therapeutic angiogenesis. Under normal physiological conditions endothelial cells undergo a low level of turnover. However, under pathological conditions this can be significantly increased with a shift in balance of proangiogenic and angiostatic factors. For example, the development of collateral circulation in the myocardium occurs naturally in tissue that is exposed to chronic mild ischaemia. These conditions induce a revascularisation process, for example, through the activity of VEGF (Lee and Feldman, 1998; Lee *et al*., 2003).

VEGF acts on endothelial cells to increase proliferation and vascular permeability (Josko *et al*., 2000). VEGF has four

isoforms 121, 159, 206 and 165 which act on tyrosine kinase receptors, FLK-1 and FT1. It can be induced in endothelial cells in response to a number of stimuli including hypoxia, interleukin (IL)-1, endothelin-1, cAMP, Ca^{2+}, steroids and cytokines including tissue growth factor, platelet-derived growth factor and fibroblast growth factor (FGF). Alternatively cGMP decreases VEGF levels as does nitric oxide (NO) (Josko *et al*., 2000). In addition to VEGF a number of other candidate genes have been suggested for use in therapeutic angiogenesis including FGF, hepatocyte growth factor (HGF) as well as developmentally regulated endothelial locus 1 (Del-1) (Giordano *et al*., 1996; Aoki *et al*., 2000; Zhong *et al*., 2003).

FGFs are a group of growth factors that are released into the extracellular matrix by cell death or damage. Their potential use in therapeutic angiogenesis has been comprehensively reviewed by Khurana and Simons (2003). FGF consists of 22 related proteins, which affect cellular growth, proliferation and migration (Ornitz and Itoh, 2001). These proteins also act via tyrosine kinase receptors in this case four high affinity receptors. FGF3, -4 and -5 are known to have significant regulatory effects on angiogenesis. FGF2 has been shown to play a role in the generation of larger vessels not just capillary buds, which may be useful in relieving chronic, stable angina. FGFs are potent endothelial cell mitogens and also serve as ligands for other cell types including vascular smooth muscle cells and fibroblasts (Ornitz and Itoh, 2001). The FGFs, such as VEGF, also stimulate endothelial cell synthesis of proteases including plasminogen activator and metalloproteinases, important for extracellular matrix digestion in the process of angiogenesis.

The strategy of therapeutic angiogenesis (Figure 2) has been used in peripheral vascular disease and coronary artery disease both in preclinical models (Table 1) and subsequently in clinical trials (Table 2). At critical points in the disease process a relatively brief expression of transgene may be sufficient which would not require efficient gene delivery, particularly if the transgene product is potent and secreted.

Peripheral vascular disease. Atherosclerosis may develop in the peripheral circulation leading to critical limb ischaemia. It is estimated to develop in 500–1000 people per million per year and with age as a risk factor has an increasing prevalence in our aging society (Hooi *et al*., 2001; Morishita, 2004). The prognosis for patients with chronic critical leg ischaemia is often poor. These patients continue to suffer from the debilitating symptoms of their disease and remain at risk for limb amputation.

Preclinical studies. The favoured model for studying peripheral vascular disease is the hindlimb ischaemia model usually in mouse, rat or rabbit models (Takeshita *et al*., 1994; Taniyama *et al*., 2001; Dai *et al*., 2002; Xie *et al*., 2006) although the pig model has also been examined (Pipp *et al*., 2004). Both viral and nonviral methods have been used to deliver transgenes to the muscle of the ischaemic limb in the various models mentioned above. Nonviral methods include direct injection of plasmid DNA as well as utilising formulations of lipids or polymers to aid DNA uptake by cells (Ledley, 1994). Various growth factors have been studied and shown to induce angiogenesis in these models including FGF, HGF, IGF and in particular VEGF which has been shown to be successful in inducing collateralisation in both rat and rabbit hind-limb ischaemia models (Tsurumi *et al*., 1996; Rasmussen *et al*., 2002; Brar *et al*., 2004). Delivery has been shown to be effective whether by direct injection of naked DNA or by adenoviral delivery. An adenoviral vector carrying the VEGF 121 isoform and driven by a CMV promoter has

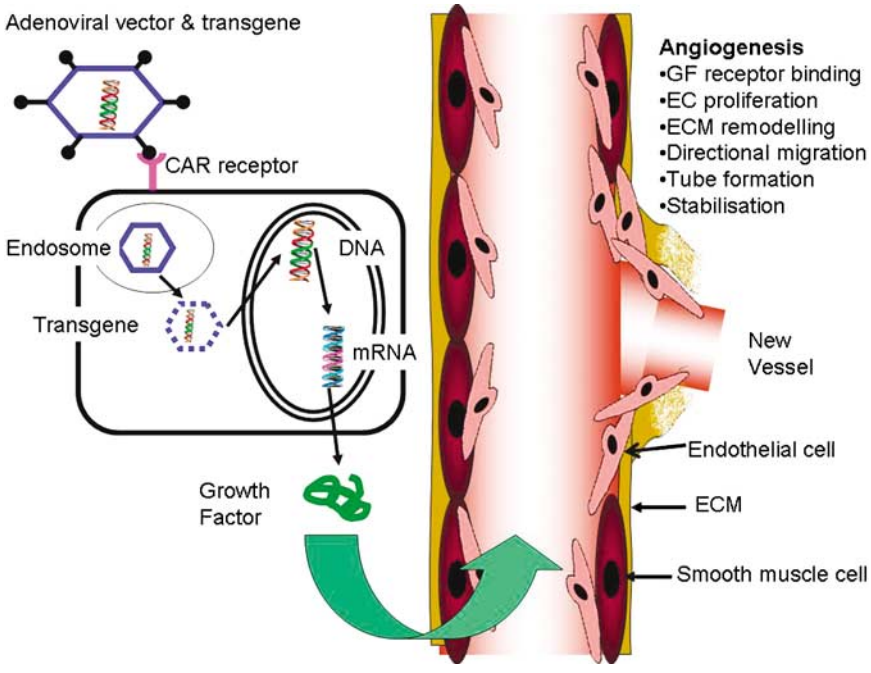

Figure 2 Therapeutic angiogenesis. An example of adenoviral delivery of growth factor transgenes to the vasculature.

Table 1 Summary of selected preclinical gene therapy studies

Target	*Model*	*Transgene*	*Study*	*End point*
Peripheral vascular disease	HLI, rabbit	VEGF and bFGF	Kondoh *et al*. (2004)	Combination effective for collaterals
	HLI, rabbit	bFGF	Ishii *et al*. (2004)	Significant collateralisation
	HLI, rabbit	VEGF165 and Ang-1	Ohara *et al*. (2001)	Significant neovascularisation
	HLI, mouse	VEGF121, -165, -189	Whitlock *et al*. (2004)	Combination effective
Myocardial ischaemia	Ameroid LCCA, pig	VEGF165	Laguens *et al*. (2002)	Increased cariomyocyte mitosis
	Ameroid LCCA, mini-swine	VEGF165	Zhang *et al*. (2002)	Decreased ischaemia area and increased collaterals
	Stress, pig	FGF4	Gao *et al*. (2004)	No toxic effects
	Ameroid LCCA pig	HIFα	Heinl-Green *et al*. (2005)	Increased perfusion
Neointimal hyperplasia	Vascular graft, pig	TIMP-3	Akowuah *et al*. (2005)	Increased luminal area
	Vascular graft, rat	Antisense TGF	Wolff *et al*. (2005)	Reduced neointimal hyperplasia
	Vascular graft, rabbit	NOS	West *et al*. (2001)	Reduced neointimal hyperplasia
	Vascular graft, pig	p53	Wan *et al*. (2004)	Reduced neointimal hyperplasia
	PTA and stent, rat	iNOS	Fishbein *et al*. (2006)	Reduced ISR
	PTA and stent, pig	TIMP-3	Johnson *et al*. (2005)	Reduced ISR
	PTCA and stent, rabbit	VEGF-2	Walter *et al*. (2004)	Reduced ISR and increased RE

Abbreviations: bFGF, basic fibroblast growth factor; HIFα, hypoxia-inducible factor α; HLI, hindlimb ischaemia; iNOS, inducible nitric oxide synthase; ISR, in-stent restenosis; LCCA, ameroid constrictor placed on the left circumflex artery; NOS, nitric oxide synthase; PTA, percutaneous transluminal angioplasty; RE, re-endothelialisation; TGF, tissue growth factor; TIMP-3, tissue inhibitor of matrix metalloproteinase-3; VEGF, vascular endothelial growth factor.

been termed the BIOBYPASS and has shown promise in preclinical studies (Basara, 2001). Other methods of delivery of VEGF and FGF include a combined gene and cell therapy method, which packages these genes *ex vivo* into autologous fibroblasts. Interestingly, when fibroblasts transduced with both genes were introduced into the rabbit ischaemic limb there was a synergistic effect observed for neovascularisation (Kondoh *et al*., 2004). Other potential angiogens have also been identified as being potentially useful for treating peripheral ischaemia including the telomerase (Zaccagnini *et al*., 2005) or the extracellular matrix protein, del-1, a potent angiogenic factor during embryogenesis (Penta *et al*., 1999; Rezaee *et al*., 2002; Zhong *et al*., 2003).

Thus far, the majority of studies, which have examined the use of gene therapy in treating ischaemic disease, have used delivery of single genes. These single genes are either at critical control points in the underlying pathology or represent the start of a cascade of effects. Other potential methods, which have been examined in preclinical models, include the use of more than one active gene or the use of transcription factor overexpression, which would result in induction of a repertoire of genes. Elegant methods of ameliorating vasculogenesis have been put forward in preclinical models using bicistronic vectors carrying VEGF as well as angiopoeitin or using multiple isoforms of VEGF in various ratios (Niagara *et al*., 2004; Whitlock *et al*., 2004; Yu *et al*., 2006). Collectively, these data suggest that revascularisation may be more efficiently achieved through complex simultaneous activation of various pathways. Potential has also been shown using *ex vivo* gene therapy of fibroblasts using adenoviral vectors (Ohara *et al*., 2001; Ishii *et al*., 2004).

In summary, a large volume of preclinical efficacy and toxicology data paved the way for human studies, which will next be reviewed.

Clinical trials. Trials involving gene therapy to stimulate angiogenesis have been undertaken in patients with peripheral vascular disease. The efficacy of therapeutic angiogenesis using VEGF gene transfer has been reported in human patients with critical limb ischaemia (Baumgartner *et al*., 1998). Initially, the use of a hydrogel catheter with naked $VEGF_{165}$ plasmid was proposed based on ischaemic hindlimb models (Isner *et al*., 1996). This procedure can effectively stimulate collateral blood vessel development in animal models; however, many patients do not have appropriate target lesions amenable to catheter-based gene delivery, so this was not considered an ideal treatment. Thus, intramuscular injection of naked plasmid encoding $VEGF_{165}$ was used (Baumgartner *et al*., 1998). This trial involved small numbers of patients and did not enrol placebo controls but did demonstrate tolerability and promising clinical efficacy for treatment of peripheral arterial disease Kim *et al*. (2004). also reported the results of a phase I clinical trial using naked plasmid DNA encoding for $VEGF_{165}$ in patients with severe debilitating peripheral vascular disease. Again the injections were well tolerated and results showed increased collateral vessel formation around the injection sites. Ischaemic pain and ischaemic ulcers in the affected limb were relieved or markedly improved in a significant number of patients but again a placebo control group was not included.

The above trials used naked plasmid DNA encoding for VEGF, however adenoviral $VEGF_{121}$ has also been investigated with similar results. Initially, Rajagopalan *et al*. (2001) reported that administration of intramuscular $AdVEGF_{121.10}$ to skeletal muscle of five patients with severe peripheral vascular disease improved endothelial function and lower extremity flow reserve.

HGF has also been examined in the context of treating peripheral ischaemia. A prospective, open-labelled clinical trial was carried out, which examined the safety and efficacy of intramuscular injections of HGF plasmid DNA in patients with chronic limb ischaemia (Morishita *et al*., 2004). This limited trial had promising initial results with the treatment reducing the pain index in 5/6 patients, increasing the ankle pressure index by >0.1 in all patients, and improving the size of ischaemic ulcers by $>25\%$ in four patients. However,

Table 2 Summary of cardiovascular gene therapy and genetic decoy-based clinical trials

DiseaseTarget	*Trans gene*	*Study*	*Vector*	*Delivery system*	*Placebo*	*No*	*Primary end points*	*Result*
CLI	HGF	Morishita *et al.* (2004)	Plasmid	IM	No	6	Pain, ABI, Ulcer	Improved
	VEGF	Baumgartner *et al.* (1998)	Plasmid	Catheter	No	9	ABI, limb salvage	Improved
	VEGF	Isner *et al.* (1996)	Plasmid	Catheter	No	1	Angiography, Doppler	Improved
PVD	VEGF	Kim *et al.* (2004)	Plasmid	IM	No	9	Angiography, ABI, pain	Improved
	VEGF	Rajagopalan *et al.* (2001)	Adenovirus	IM	No	5	Endothelial activity	Improved
Intermittent claudification	VEGF	Rajagopalan *et al.* (2003) (RAVE)	Adenovirus	IM	Yes	105	Peak walking time	NSD
	VEGF	Kusumanto *et al.* (2006)	Plasmid	IM	Yes	27	Amputation rate at 100 days	NSD
Angina	VEGF	Losordo *et al.* (2002)	Plasmid	Catheter	Yes	19	CCS angina class	Improved
		Fortuin *et al.* (2003)	Plasmid	Thoracotomy	No	5	CCS angina class	Improved
	FGF	Grines *et al.* (2002) (AGENT I)	Adenovirus	Catheter	Yes	79	ETT	Trend
		Grines *et al.* (2003) (AGENT II)	Adenovirus	IC injection	Yes	52	Reversible perfusion defect size	Trend
VBG	E2F decoy	Mann *et al.* (1999) (PREVENT I)	DNA	Instilled	Yes	41	Safety and occlusions	Trend
		Grube *et al.* (2001) (PREVENT II)	DNA	Instilled	Yes	200	Occlusion, vessel wall thickness	Improved (30%)
		Conte *et al.* (2005) (PREVENT III)	DNA	Instilled	Yes	1404	Reintervention	NSD
		Alexander *et al.* (2005) (PREVENT IV)	DNA	Instilled	Yes	3014	Angiographic failure	NSD
ISR	VEGF	Laitinen *et al.* (2000)	PL/Lipo	I/P catheter	No	15	Safety, angiography	NSD
		Hedman *et al.* (2003)	PL/Lipo/Ad	I/P catheter	No	103	Angiography, restenosis	NSD
	(KAT I)						Myocardial perfusion	

Abbreviations: ABI, ankle-brachial index; Ad, adenovirus; CCS, Canadian Cardiovascular Society; CLI, chronic limb ischaemia; ETT, excercise treadmill test; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; I/P, infusion-perfusion; IC, intracoronary; IM, intramuscular; ISR, in-stent restenosis; Lipo, liposome; NSD, no significant difference; PL, plasmid; PVD, peripheral vascular disease; VBG, vascular bypass graft; VEGF, vascular endothelial growth factor.

as acknowledged by the authors, the small numbers and lack of a control group prevented meaningful conclusions being made regarding efficacy although short-term tolerability of the treatment was observed.

In contrast to the promising data emanating from preclinical and phase I studies, the Regional Angiogenesis with Vascular Endothelial Growth Factor trial (RAVE Trial), which was reported in 2003, demonstrated a less optimistic outcome using intramuscular $AdVEGF_{121}$ (Rajagopalan *et al*., 2003). This trial was a phase II, randomised, double-blind, placebo-controlled trial examining the safety and efficacy of administration of intramuscular VEGF 121 isoform to the lower extremities of patients with disabling unilateral intermittent claudication. The outcome of this study demonstrated that administration of $AdVEGF_{121}$ was not associated with improved exercise performance or quality of life. A similar lack of efficacy for a primary endpoint in preventing amputations has been observed in placebo-controlled trials where plasmid $VEGF_{165}$ was delivered intramuscularly to diabetic patients with critical limb ischaemia. However, ulceration rates and other clinical end points were reduced (Kusumanto *et al*., 2006).

Coronary artery disease. At present the accepted method of treatment for coronary artery disease is by pharmacotherapy, risk factor modification or through invasive interventions. These interventions include percutaneous transcoronary angioplasty (PTCA) with or without stenting or coronary artery bypass grafting (CABG). Patients may fail to respond to risk factor modification or pharmacotherapy. In addition, recurrent coronary ischaemia may occur, subsequent to either intervention, due to graft failure or in-stent restenosis. These patients, refractory to current therapies, would be the main targets for gene therapy approaches to treatment initially. However, it is evident that once such therapies become more accepted that a broader patient population may then be amenable to treatment using these strategies.

Preclinical studies

Gene therapy treatment of myocardial ischaemia is a more difficult proposition than peripheral ischaemia due to issues of access and delivery of the vector. To investigate putative gene therapies, it is common to use larger animal models (Hughes *et al*., 2003) including the pig (Laguens *et al*., 2002; Zhang *et al*., 2002; Grines *et al*., 2003a; Gao *et al*., 2004; Heinl-Green *et al*., 2005; Horvath *et al*., 2005). However, there are limitations to the animal data generated because it is difficult to mimic the human condition of chronic stable ischaemia and easier to induce an acute injury through a myocardial infarction.

Similarly to peripheral ischaemia models both VEGF (Laguens *et al*., 2002; Zhang *et al*., 2002) and FGF (Grines *et al*., 2003a; Gao *et al*., 2004; Horvath *et al*., 2005) and in particular FGF-4 are popular targets for gene therapy to treat chronic and acute myocardial ischaemia. Results using these genes have been promising in animal models as have other molecular targets including transcription factors and the master-switch HIF-1α (Shyu *et al*., 2002; Heinl-Green *et al*., 2005).

Studies have also examined methods to improve the efficiency of gene delivery to the diseased myocardium. An elegant method has been identified to express transgenes only under appropriately hypoxic conditions to avoid pathological angiogenesis (Tang *et al*., 2002, 2005). This is achieved using a hypoxia-inducible VEGF gene therapy system using the erythropoietin enhancer and a water-soluble lipopolymer or alternatively using a double plasmid system. Cardiomyocytes may also be intrinsically protected from hypoxic damage by the induction of Akt, which may aid myocyte survival, through upstream overexpression of insulin-like growth factor-1 (Chao *et al*., 2003). An exciting area of advancement in the field of gene therapy is the combined use of both gene therapy with stem cell therapy in particular the use of bone marrow-derived mesenchymal stem cell (MSC) lines. These pluripotent cells can differentiate into myocytes, but have only demonstrated minor improvement in the function of damaged myocardium when transplanted in a pig model (Shake *et al*., 2002). However, the work of Mangi *et al*. (2003), and Noiseux *et al*. (2006) has demonstrated that overexpression of Akt in MSCs *ex vivo*, significantly improves the effects of this approach in restoring myocardial function *in vivo* after ischaemic damage. It is probable that such effects are most likely paracrine in nature rather than due to an enhanced survival of MSCs *in situ* conferred by Akt.

Clinical trials

The system for vector delivery assumes considerable importance in the heart. The various studies reviewed below have examined methods of direct injection to the myocardium through a mini-thoracotomy or in conjunction with CABG (Rosengart *et al*., 1999b) or through infusion from an intracoronary catheter or cardiac navigation system allows system which is direct intramyocardial delivery to viable areas of myocardium (Kastrup *et al*., 2005).

A study by Losordo *et al*. (2002) examined the use of naked plasmid DNA encoding VEGF delivered directly to the myocardium by injection through a mini-thoracotomy. The patients had stable chronic angina refractory to current treatments with Canadian Class 3–4 angina. Symptomatic relief in all five patients was observed between 10 and 30 days later suggesting that gene expression had reached significant levels. All patients had improved anginal status and were able to decrease medication use. In addition, harder end points such as myocardial perfusion assessed using SPECT-sestambi and angiography, were improved in all patients. However, only a small number of patients were used (five) in this study and it was not placebo-controlled. A similar phase I study was carried out by direct myocardial injection in combination with CABG and demonstrated promising trends regarding excercise treadmill test (ETT) and anginal class at 6-month follow-up (Rosengart *et al*., 1999a). A subsequent study by Fortuin *et al*. (2003) delivered naked plasmid DNA via a thoracotomy. At 1-year follow-up similarly encouraging results were seen in 30 patients with refractory angina. Improved ETT times, anginal class and decreased frequency of medication use were observed in comparison to baseline and this peaked at 6 months. Follow-up

persisted to 12 months and as reported by Reilly *et al*. (2005), out to 24 months. Dose escalation was used in the study but the improvements were not dose-dependent. However, the low numbers and lack of a placebo control group limit the usefulness of the data generated.

Based on the promising results using recombinant FGF in a phase I trial (Schumacher *et al*., 1998a, 1998b), the first study to use adenovirus to deliver FGF was carried out (AGENT 1) (Grines *et al*., 2002). This study used catheters to deliver Ad5-FGF4 to the myocardium of 79 angina patients. The trial was a multicentre, double-blind, randomised, placebo-controlled study. It aimed again to establish safety in the patient population and also to assess some pharmacokinetic data such as vector distribution. In the latter case, the vector was distributed to the pulmonary circulation but was not excreted in the urine nor was any evidence of it found in germ cells using PCR. It should be noted that FGF4 binds to the proteoglycans of the cells it is secreted by and therefore may have less systemic effects. No retinopathy, angioma or myocarditis was observed. Notable again in two cases a malignancy was unmasked during the trial. The first was a colorectal cancer, which metastasised but was more likely due to hereditary nonpolyposis colorectal cancer owing to family history. Ad5-FGF4 could not be detected in the neoplasm. Another patient developed a glioblastoma. An improved ETT was noted in treated groups compared with baseline values but when compared with placebo controls this was not significant. The study was discontinued as enough safety data had been generated to design AGENT2 (Grines *et al*., 2003b). This subsequent study had 52 patients and examined dose escalation and looked at reversible perfusion defect size as an end point. Again the active treatment group had no significant benefit when compared to controls.

As comprehensively reviewed by Isner *et al*. (2001) the relative safety of angiogenic gene therapy and in particular delivery of the VEGF gene to the myocardium has been repeatedly demonstrated. Studies using this approach have a low number of significant adverse events compared with the general population. Of concern are the theoretical risks concerning the effect angiogenesis may have on accelerating disease states already present in the patient. It has been suggested that angiogenesis could contribute to an acceleration of atherosclerotic disease due to increased perfusion of fibrofatty plaques and it has been shown experimentally that inhibition of plaque angiogenesis can reduce the growth of atherosclerotic lesions. However, gene delivery of angiogenic agents such as VEGF has not been shown to have an adverse effect on atherosclerotic lesions in current phase I trials. This is most likely due to the relatively localised and low levels of angiogenic agents involved. Other potential adverse outcomes for angiogenic-based gene therapy include the development of vascular malformations, diabetic retinopathy and oedema. Again the occurrence of these adverse effects has been rare in patients undergoing gene therapy. It has also been suggested that angiogenic gene therapy could potentially contribute to the progression of neoplasia by contributing to the vascularisation of an existing primary mass aiding growth and metastatic spread. However, in the present short-term studies, which have been conducted an increased risk of neoplasia has not been observed. However, some adverse effects may not become apparent until larger and longer-term studies have been conducted with effective monitoring and reporting of any such outcomes.

Summary. Thus far, the promise shown by preclinical studies of therapeutic angiogenesis using gene therapy has not been matched by clinical trial results. Even in cases where efficacy has been noted (Losordo *et al*., 2002; Fortuin *et al*., 2003), the inclusion of a placebo control group invariably eliminates this difference. This may be due to the fact that multiple genes may be required to establish a suitably angiogenic local environment. Single genes have been used to date and the use of vectors with a larger capacity such as herpes simplex virus type-1 may allow multiple genes to be expressed to provoke an angiogenic environment (Epstein, 2005). Alternatively, the most useful gene may not yet have been discovered. The time course of expression may also be very important and prolonged low-level expression may be more suitable to establish efficacy. Another issue may be the route of vector delivery. For example, in the AGENT trial, the vector was delivered by an intracoronary route, whereas a more direct delivery to the myocardium using the minimally invasive NOGA system may be more effective method of delivering genes to the heart (Kastrup *et al*., 2005). Finally, the extent of angiogenesis observed in the placebo group in phase II studies is a significant issue, which should be considered in the design of future trials in this area.

Several positive features have been elucidated from the clinical data to date in the area of biosafety and tolerability (Rosengart *et al*., 1999a, 1999b). Therapeutic angiogenesis is a theoretically perilous strategy considering the potential risks associated with unrestrained blood vessel formation in the eye and in particular in the area of malignancies. The studies to date, however, have suggested that the strategy is safe without an increased rate of adverse outcomes in the active treatment groups.

Prevention of neointimal hyperplasia

Introduction. The development of neointimal hyperplasia contributes to the pathogenesis of both vein graft failure and in-stent restenosis (Figure 3). Neointimal graft wall thickening is an adaptive reaction that provides haemodynamic stability to the graft. However, this leads to the upregulated expression of proinflammatory growth factors, cytokines and adhesion molecules by the neointimal vascular smooth muscle cells. The resulting inflammation results in dysfunction of the overlying endothelium.

Gene therapy offers a potential role in this setting by targeting:

1. The modulation of smooth muscle cell migration
2. The modulation of smooth muscle cell proliferation
3. Smooth muscle cell apoptosis
4. The acceleration of re-endothelialisation and improvement of endothelial function.

A number of potentially therapeutic genes, which are key to neointimal formation, have been identified (Baker, 2002).

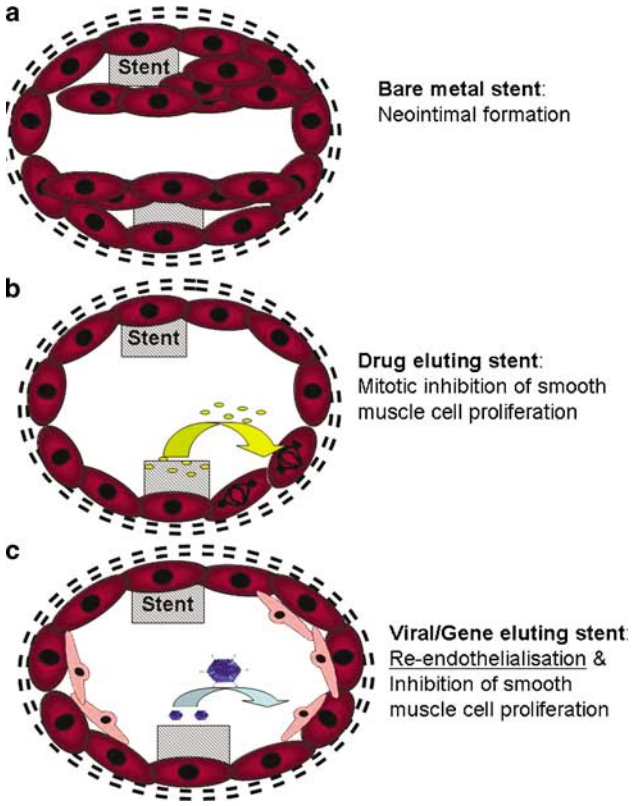

Figure 3 Inhibition of in-stent restenosis (**a**) using drug-eluting stents (**b**) and viral/gene-eluting stents (**c**).

These include tissue inhibitors of matrix metalloproteinases (TIMP-3) (Akowuah *et al*., 2005), nitric oxide synthase (NOS) (West *et al*., 2001) and p53 (George *et al*., 2001; Wan *et al*., 2004). The use of E2F decoy oligodeoxynucleotides to modulate gene expression is not strictly gene therapy but this related approach will be also be discussed here. A summary of preclinical and clinical trials using these therapeutic approaches can be seen in Tables 1 and 2, respectively.

Vein graft failure. Direct access to the vein during surgery offers a major potential benefit as a target for gene therapy. The vein graft can be manipulated and therapeutic genes, which may prevent subsequent graft failure can be applied *ex vivo*.

Preclinical studies

Overexpression of TIMPs prevents the digestion of the extracellular matrix by matrix metalloproteinases, which is required for smooth muscle cell migration. Studies involving the overexpression of TIMP 1–3 in human saphenous vein cell cultures *ex vivo*, demonstrated inhibition of migration of smooth muscle cells thus reducing neointimal formation. TIMP-3 blocks neointimal formation *in vivo* in pig grafts at 28 days post-surgery (Akowuah *et al*., 2005). TIMP-3 is thought to work best because of its ability to bind to the extracellular matrix, induce smooth muscle cell apoptosis and block migration of smooth muscle cells.

Other approaches, which have proven successful in various animal models of neointimal hyperplasia in vein grafts (Petrofski *et al*., 2004), include successful attempts to downregulate the expression of chemotactic and mitogenic factors in the graft or to inhibit the signalling cascade responsible for neointimal hyperplasia (Wolff *et al*., 2005).

Studies by West *et al*. (2001) have shown that Adenoviral mediated gene transfer of neuronal NOS (nNOS) can improve endothelial cell function as well as blocking smooth muscle cell proliferation following vein bypass grafting . They demonstrated that NOS gene transfer intraoperatively in vein grafts can result in reduced endothelial activation and inflammation. In mature vein grafts this leads to decreased smooth muscle cell intimal hyperplasia. Furthermore, overexpression of NOS during the early phase of vein graft remodelling results in intimal smooth muscle cells which are modulated toward a more differentiated phenotype with reduced smooth muscle cell-related superoxide production. This suggests that both reduced smooth muscle cell proliferation and intimal hyperplasia may be achieved through increased production of NO. Furthermore, such an increase in NO can also result in a reduction in superoxide production thus protecting the vein graft from damage by reactive oxygen species. It may be that early and transient increases in NO can result in long-term benefits, which do not require persistent overexpression of NOS making vein grafts a potential target for gene delivery methods intraoperatively to prevent vein graft failure. This approach to preventing neointimal formation has been further confirmed by *in vivo* experiments comparing other NOS isoforms (Cooney *et al*., 2006).

It has been shown that wild-type p53 gene transfer inhibits neointimal formation in human saphenous vein segments by modulation of smooth muscle cell migration and induction of apoptosis (George *et al*., 2001). The same group also studied adenoviral-mediated gene transfer of p53 in porcine interposition vein grafts and found that it resulted in increased lumen size and inhibition of neointimal formation (Wan *et al*., 2004).

Antisense oligonucleotides that block cell-cycle gene expression can inhibit experimental neointimal hyperplasia, stabilise vein graft endothelial function and prevent graft atherosclerosis (Morishita *et al*., 1995). The more cell-cycle regulatory genes blocked by an agent, the more effective is the therapy. Thus, an ideal target for cell-cycle blockade is E2F, a transcription factor that leads to upregulation of up to 12 cell-cycle genes. A strategy to block E2F using a double-stranded decoy oligodeoxynucleotide that bears the consensus E2F-binding site (E2F decoy oligodeoxynucleotides) is a good single agent strategy for prevention of vein graft disease. *Ex vivo* studies in rabbits have shown that vein grafts treated intraoperatively with E2F decoy oligodeoxynucleotides resulted in inhibition of neointimal hyperplasia and graft atherosclerosis for up to 6 months.

Clinical trials

On the basis of potential shown of this approach to block cellular proliferation using E2F decoy oligodeoxynucleotides, the PREVENT Trial entered a phase 1 trial. This was a single-centre, prospective, randomised controlled trial of *ex vivo* therapy for human vascular bypass grafts which

manipulated gene expression using an E2F decoy oligodeoxynucleotide (Mann *et al*., 1999). There was a 74% reduction in vascular cell proliferation in the E2F decoy oligodeoxynucleotide treated group. This group also displayed fewer graft occlusions at 30 days compared with the untreated group (11.8 and 25%, respectively) as well as fewer nontechnical primary failures among high-risk patients. Similar safety and tolerability results were observed for the PREVENT II trial, which used this gene manipulation strategy for coronary bypass grafts (Grube, 2001). Although the phase 1 trial (PREVENT Trial) showed promising results, subsequent studies were less positive including recently published results of the phase III, multicentre, randomised double-blinded, placebo-controlled trial of 3014 patients undergoing primary CABG surgery with at least two planned saphenous grafts and without concomitant valve surgery (Alexander *et al*., 2005; Conte *et al*., 2005, 2006). The vein grafts were treated *ex vivo* with either edifoligide (E2F decoy oligodeoxynucleotide) or placebo. The trial showed that edifoligide had no effect on vein graft failure compared to the placebo-treated group (45.2 vs 46.3%, respectively).

In-stent restenosis. As previously mentioned, stented vessels may undergo restenosis in up to 30% of patients, 6 months post-PTCA with stenting (Rome *et al*., 1994). To prevent this complication, drug-eluting stents have been used to prevent vascular smooth muscle proliferation, which is the major contributor to in-stent restenosis. These stents have a polymer coating capable of stably eluting anticoagulants or antimitotic drugs. However, drug-eluting stents also inhibit re-endothelialisation of the stented segment making it liable to thrombosis. Furthermore, although this method tackles the physical blockade of the stent it does not address the underlying pathology of the disease process. Therefore, it may become attractive to combine stenting with gene delivery to both prevent in-stent restenosis and to allow a stable platform for gene delivery over time which may ameliorate the disease process.

Preclinical studies

The initial concept of using genetically modified organisms to help prevent in-stent restenosis was published by Dichek *et al*. (1989), who established that genetically altered endothelial cells overexpressing, human-type tissue plasminogen activator or reporter transgenes could be seeded and grown onto intravascular stents (Koren *et al*., 2006). To examine in-stent restenosis larger animal models are generally used including the rabbit, pig and monkey (Sharif *et al*., 2004). A rodent model is also in use (Fishbein *et al*., 2006). Studies have focused on establishing proof of principle for various genes as well as the technical aspects of when and how to deliver the genes from the stent or via a catheter. Numerous genes have shown promise in reducing in-stent restenosis in animal models. These include VEGF, inducible NOS (iNOS) and TIMPs, which have been effective in animal models (Sharif *et al*., 2004). The development of stents, which elute genes directly via plasmids or adenoviral vectors, has proven to be an effective means of delivering transgenes (Walter *et al*., 2004; Johnson *et al*., 2005; Sharif *et al*., 2006). However, such direct gene-eluting stents have yet to be examined in human trials with catheter-based infusion techniques being favoured thus far (Hedman *et al*., 2003).

Clinical trials

Once again VEGF was the initial anti-restenotic gene chosen for use due to its known effect in inducing NO and prostacyclin, which both inhibit VSMC migration and proliferation. Laitinen *et al*. (2000) examined a small number of patients (15) undergoing PTCA or PTCA with stenting. VEGF was delivered using plasmid/liposome DNA subsequent to and at the site of the PTCA procedure but before any stenting procedure. The gene transfer involved infusion of vector for 10 min through an infusion–perfusion catheter. This study used reporter genes in a control group and found the procedure to be well tolerated with little or not adverse effects but with no benefit on restenosis rates (Laitinen *et al*., 2000). Although long term (up to 180 days) and localised gene delivery was observed, the lack of beneficial or adverse effects may be due to the low efficiency gene delivery in this trial.

A subsequent study based on the results of Laitinen *et al*. (2000) was termed the Kuopio Angiogenesis Trial (KAT) and this also used an infusion–perfusion catheter. This examined the safety and feasibility of catheter-based gene delivery in 103 patients (Hedman *et al*., 2003). The study was a double-blind, randomised, placebo-controlled study. In addition to controls and plasmid/liposome-mediated delivery of VEGF, a third group had VEGF delivered using an adenovirus. The treatment was again well tolerated with serious adverse effects similar to those in the general population. However, at 6 months follow-up no difference was noted in lumen diameter within the stent using angiography, although a low overall rate of restenosis was observed in the study (6%). Also of note, an increased myocardial perfusion was observed at 6 months follow-up in the AdVEGF-treated group. This may be due to the prolonged expression of VEGF from adenoviral delivery and its more potent effect on angiogenesis than on NO induction.

Summary. It would seem likely that the effective long-term prevention of in-stent restenosis using vascular gene therapy is an achievable goal, although to date clinical results have not reflected this. It may be that the impetus in developing such strategies using gene therapy has not been great enough particularly in the era of drug-eluting stents (Burt and Hunter, 2006). However, it is clear from longer-term studies examining drug-eluting stents that late complications such as thrombosis are a significant problem with that therapy (Joner *et al*., 2006). Strategies to prevent neointimal hyperplasia while ensuring the integrity of the endothelium may be achievable using stent-based gene delivery and this is an area worthy of further study. As there is no effective means of preventing vein graft failure gene therapy approaches to this condition again focusing on genes, which may inhibit intimal hyperplasia while preserving the endothelium, would be attractive. The exciting, emerging technology of small interfering RNA which earned its

discoverers, Professor Mello and Professor Fire the 2006 Nobel prize in Physiology and Medicine may also become a more attractive gene-based therapeutic strategy in the future for cardiovascular disease processes. The technology can down-regulate activated genes associated with pathological cascades and has been shown to successfully target genes associated with vein graft failure by preventing neointimal hyperplasia in an *in vivo* model (Banno *et al*., 2006).

Future perspectives

Gene-based therapy for patients with refractory or recurrent cardiovascular disease has been the subject of extensive investigation. Although efficacy has been demonstrated in animal models and safety in phase I human studies, unequivocal evidence of efficacy has not been demonstrated in placebo-controlled trials. (Simari and O'Brien, 2002; Yla-Herttuala *et al*., 2004). The reason for this is not clear but future approaches will require optimisation of vectors for gene delivery, use of alternative genes and/or families of genes and the use of optimal *in vivo* gene delivery systems, which will result in efficient gene delivery to the target tissues. These approaches will need careful evaluation in animal models followed by appropriate clinical translation. From experience to date, evidence of efficacy should be examined in properly designed placebo-controlled clinical studies. Gene therapy approaches are particularly relevant in conditions such as 'the no-option revascularisation patient' or vein bypass graft failure where current alternative therapies are suboptimal. They may also be relevant in the prevention of in-stent restenosis if reduction of intimal hyperplasia can be prevented while maintaining endothelial integrity. In addition, gene therapy approaches to cardiovascular disease will have to demonstrate advantages over conventional therapies, which make the above-mentioned disease targets attractive.

Conflict of interest

The authors state no conflict of interest.

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