Experimental Physiology – *Themed Issue Cardiovascular Genomics Paper*

Does gene therapy become pharmacotherapy?

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Recent progress in molecular and cellular biology has led to the development of numerous effective cardiovascular drugs. However, there are still a number of diseases for which no known effective therapy exists, such as peripheral arterial disease, ischaemic heart disease, restenosis after angioplasty, and vascular bypass graft occlusion. Currently, gene therapy is emerging as a potential strategy for the treatment of cardiovascular disease despite its limitations. The first human trial in gene therapy for cardiovascular disease was started at 1994 to treat peripheral vascular disease using vascular endothelial growth factor (VEGF). Then, many different potent angiogenic growth factors were tested in clinical trials to treat peripheral arterial disease and ischaemic heart disease. Improvement of clinical symptoms in peripheral arterial disease and ischaemic heart disease has been reported. This review focuses on the future potential of gene therapy for the treatment of cardiovascular disease. In the future, gene therapy might become a real pharmacotherapy to treat cardiovascular disease.

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Currently, gene therapy is emerging as a potential strategy for the treatment of cardiovascular disease to treat peripheral arterial disease, ischaemic heart disease, vascular bypass graft occlusion and transplant coronary vasculopathy for which no known effective therapy exists. Although current *in vivo* methods for cardiovascular gene transfer are still limited by the lack of efficiency and potential toxicity, recent advances in *in vivo* gene transfer may provide the opportunity to treat cardiovascular diseases such as peripheral arterial disease by manipulating angiogenic growth factor genes. The present promising gene therapy is mainly locally administered agents, while most pharmacotherapy is based on oral drugs. To consider the advantage of gene therapy, one might compare the recombinant therapy, as both concepts are relatively closed. What are the advantages of gene therapy? First, it has the potential to maintain an optimally high and local concentration over time. This issue may be critical in the case of arterial gene therapy. However, in the case of therapeutic angiogenesis as discussed later, it may be preferable to deliver a lower dose over a period of several days or more from an actively expressed transgene in the iliac artery, rather than a single or multiple bolus doses of recombinant protein, to avoid side effects. In addition, the feasibility of a clinical trial of recombinant protein is currently limited by the lack of approved or available quantities of human quality of recombinant protein, due in large part to the nearly prohibitive cost of scaling up from research grade to human quality recombinant protein. Indeed, the report to compare the effectiveness of fibroblast growth factor-2 (FGF-2) as protein and as naked plasmid DNA in a porcine model of chronic myocardial ischaemia demonstrated that intramyocardial injection of FGF-2 plasmid was more effective than of FGF-2 protein in improving regional perfusion and contractility compared to untreated ischaemia (Heilmann *et al*. 2002). In contrast, gene therapy also has disadvantages such as safety aspects, and local and limited effects. After overcoming these disadvantages, gene therapy might become a real pharmacotherapy.

Gene therapy to treat peripheral arterial disease using therapeutic angiogenesis

Critical limb ischaemia that is estimated to develop in 500–1000 individuals per million per year is considered one of the most suitable diseases for gene therapy. Recently, the efficacy of therapeutic angiogenesis using vascular endothelial growth factor (VEGF) gene transfer has been reported in human patients with critical limb ischaemia (Isner *et al*. 1996*a*, 1998; Baumgartner *et al*. 1998, 2000). An initial trial was performed using a hydrogel catheter with naked VEGF165 plasmid in 1994 by Professor Isner. Although this procedure seems to be effective to stimulate

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collateral formation in patients with peripheral arterial disease (Isner *et al*. 1996*a*, 1998; Baumgartner *et al*. 1998, 2000), it is not ideal to treat many patients, as most patients lack an appropriate target vascular lesion for catheter delivery. Thus, intramuscular injection of naked plasmid encoding *VEGF165* gene was used (Fig. 1). This clinical trial demonstrated clinical efficacy for treatment of peripheral arterial disease (Isner *et al*. 1996*a*, 1998; Baumgartner *et al*. 1998, 2000). Since then, numerous angiogenic growth factors such as VEGF121, VEGF-2 and bFGF (fibrolast growth factor) have been tested in clinical trials (Makinen *et al*. 2002; Comerota *et al*. 2002). In addition to intramuscular injection of naked plasmid DNA, adenoviral delivery, liposomal delivery of angiogenic growth factors was also utilized in these trials (Isner *et al*. 1996*a*, 1998; Baumgartner *et al*. 1998, 2000; Rajagopalan *et al*. 2001; Makinen *et al*. 2002; Comerota *et al*. 2002; Rajagopalan *et al*. 2003), despite an unfortunate accident at the University of Pennsylvania (Morishita *et al*. 1999). A study using adenovirus encoding *VEGF121* demonstrated the improvement of endothelial dysfunction in response to acetylcholine or nitroglycerine (Rajagopalan *et al*. 2001). In addition to the intramuscular injection of VEGF165 and VEGF2 plasmid DNA, a trial of local catheter-mediated *VEGF165* gene therapy in ischaemic lower-limb arteries after percutaneous transluminal angioplasty (PTA) was also reported (Comerota *et al*. 2002). However, the high level of incidence of oedema as a side effect has been reported in a VEGF trial. In the case of Fontaine II as intermittent claudication, the recent result from the Regional Angiogenesis with Vascular Endothelial growth factor (RAVE) trial as the randomized study of adenoviral vascular endothelial growth factor *VEGF121* gene transfer was not successful (Rajagopalan *et al*. 2003). The selection of the agent (*VEGF121 versus VEGF165*), patient population (intermittent claudication *versus* critical limb ischaemia) and outcome measures (peak walking time *versus* ulcer size) should be considered in the quest for optimal angiogenic strategies that result in the growth of functional blood vessels and improvement in clinical symptoms.

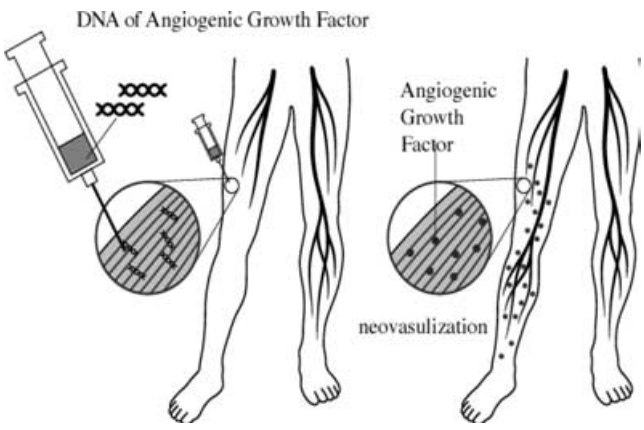

Figure 1. Concept of therapeutic angiogenesis using DNA of angiogenic growth factors

The safety and efficacy of increasing single and repeated doses of intramuscular naked plasmid DNA encoding for FGF type 1 administered to patients with unreconstructible end-stage peripheral arterial disease (PAD) was also reported (Makinen *et al*. 2002). A significant reduction in pain and aggregate ulcer size was detected after *FGF* gene transfer associated with an increased transcutaneous oxygen pressure and ankle pressure index (ABI) as compared with baseline pretreatment values (Makinen *et al*. 2002). We and others also identified hepatocyte growth factor (HGF) as a novel candidate for therapeutic angiogenesis (Nakamura *et al*. 1996; Belle *et al*. 1998; Morishita *et al*. 1999; Hayashi *et al*. 1999; Taniyama *et al*. 2001*a*,*b*; Morishita *et al*. 2002; Hiraoka *et al*. 2003; Koike *et al*. 2003). Based upon these findings, we planned a human clinical trial using intramuscular injection of naked human HGF plasmid (0.5 mg × 4 or 8 sites) twice every month. Currently, *HGF* gene transfer has been performed in 22 patients with PAD or Buerger disease of Fontaine grade IIb, III or IV who had failed conventional therapy. Preliminary results demonstrated the significant reduction in pain and increase in ABI (Morishita *et al*. 2004). Of importance, the serum level of human HGF protein did not change during gene therapy. It is noteworthy that there was no evidence of oedema in the patients transfected with the human HGF gene, in marked contrast to the VEGF trial in which 60% of patients developed moderate or severe oedema in a phase I/IIa trial. Currently, a phase III trial in Japan and phase II in the USA to treat PAD are underway.

Gene therapy to treat myocardial ischaemic disease using therapeutic angiogenesis

Professor Isner and colleagues have applied a similar idea to treat coronary artery disease using *VEGF165* gene (Losordo *et al*. 1998; Vale *et al*. 2000). An initial trial was performed with intramuscular injection of naked plasmid encoding *VEGF* gene into ischaemic myocardium through mini-operation. They reported that the transfection of *VEGF* gene resulted in a marked increase in blood flow and improved clinical symptoms without apparent toxicity (Losordo *et al*. 1998; Vale *et al*. 2000). These data revealed that *phVEGF165* (human *VEGF165* plasmid DNA) gene therapy may successfully rescue foci of hibernating myocardium. In addition, a phase 1 clinical trial of direct myocardial gene transfer of naked DNA-encoding VEGF165, as sole therapy for refractory angina, was reported in 30 patients with class 3 or 4 angina (Lathi *et al*. 2001; Fortuin *et al*. 2003). Similarly, gene therapy using *VEGF121* gene was performed by intramuscular injection of adenoviral vector (Rosengart

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Table 1. Clinical trial of angiogenesis gene therapy

Company	Gene	Targets	Phase
Vascular Genetics	VEGF-2	PAD	IIb
		CAD	IIb
GeneVec	VEGF-121	CAD	IIb
Collateral/Shering	FGF-4	CAD	IIb/III
AnGes	HGF	PAD	III (Japan)
		PAD	II (USA)
		CAD	I (USA)
Genzyme	HIF-1	PAD	I/IIa
Gencell/Aventis	FGF	PAD	I/IIa

et al. 1999*b*). Currently, no evidence of systemic or cardiac-related adverse events related to vector administration was observed up to 6 months after therapy (Rosengart *et al*. 1999*a*). Intracoronary gene transfer of *VEGF165* resulted in a significant increase in myocardial perfusion, although no differences in clinical restenosis rate or minimal lumen diameter were present after the 6-month follow-up (Hedman *et al*. 2003). More recently, intracoronary infusion of adenovirus encoding *FGF* gene was performed in a multicentre trial as phase I/IIa. The report documented that intracoronary infusion of *FGF* gene improved cardiac dysfunction without severe toxicity (Grines *et al*. 2003). In addition, the report of the treatment of 52 patients with stable angina and reversible ischaemia documented that FGF-4 adenoviral injection resulted in a significant reduction of ischaemic defect size (Grines *et al*. 2003). However, a more recent report documented the failure of this trial (Adis International, 2002).

In addition to these angiogenic growth factors, over-expression of HGF was also reported to stimulate angiogenesis and collateral formation in a rat and canine myocardial infarction model (Ueda *et al*. 1999; Aoki *et al*. 2000). Interestingly, an antifibrosis action of HGF has been identified, as HGF inhibited collagen synthesis through transforming growth factor (TGF-β) and stimulated collagen degradation through up-regulation of matrix metalloprotease (MMP-1) and urokinase-type-plasminogen activator (uPA) (Taniyama *et al*. 2002*a*). Currently, a phase I study using HGF plasmid DNA to treat patients with severe stable angina is on-going in the USA. Overall, the treatment for coronary artery disease may be curable using therapeutic angiogenesis by gene therapy. Table 1 summarizes the present status of gene therapy trials.

Gene therapy for restenosis after angioplasty and graft failure

Another important disease potentially amenable to gene therapy in cardiovascular disease is restenosis after angioplasty. One of the attractive possibilities of treating restenosis is to inhibit target gene expression (Fig. 2). In particular, the application of DNA technology such as antisense strategy to regulate the transcription of disease-related genes *in vivo* has important therapeutic potential. Accordingly, inhibition of other proto-oncogenes such as *c-myc* by antisense oligodeoxynucleotidase (ODN) was also reported to inhibit neointimal formation in several animal models (Shi *et al*. 1994). Currently, a phase II trial using antisense c-myc to treat restenosis is underway. However, as this trial utilized intracoronary infusion of antisense c-myc ODN, several issues such as low transfection efficiency may limit the efficacy of this strategy. In addition, transfection of *cis*-element double stranded (ds) ODN (= decoy) has been reported as a powerful tool in a new class of antigene strategies for gene therapy (Morishita *et al*. 1998; Fig. 3). Transfection of ds ODN corresponding to the *cis* sequence will result in the attenuation of authentic *cis–trans* interaction, leading to the removal of *trans*-factors from the endogenous *cis*-element, with subsequent modulation of gene expression. Therefore, the decoy approach may also enable us to treat diseases by modulation of endogenous transcriptional regulation. Transfection of decoy ODN against E2F, a key transcription factor for cell cycle progression, into rat and porcine balloon-injured arteries resulted in the inhibition of neointimal formation after balloon injury (Morishita *et al*. 1995; Nakamura *et al*. 2002). Based on these results, we started a clinical trial using hydrogel catheter delivery of E2F decoy to treat restenosis after angioplasty in April 2000. We did not observe any side effects up to 6 months after delivery, although the clinical outcome has not yet been evaluated.

In addition, clinical application of 'decoy' against E2F was also approved by the US Food and Drug Administration (FDA) to treat neointimal hyperplasia in vein bypass grafts, which results in failure in up to 50% of grafts within a period of 10 years. A proof-of-concept study, the Project in Ex-Vivo Vein Graft Engineering Via

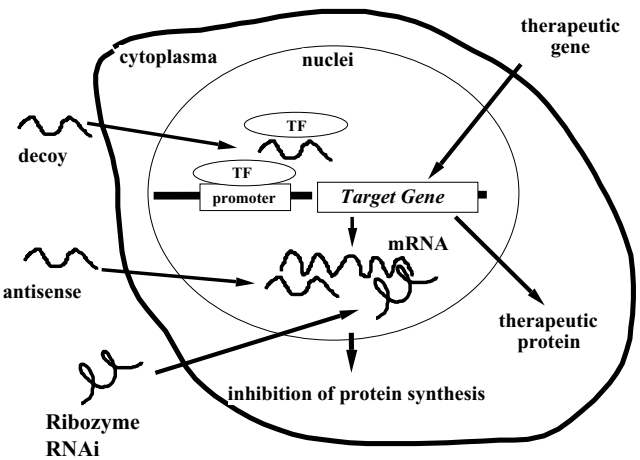

Figure 2. Target sites for antisense, ribozyme, RNAi and decoy strategies
Antisense, antisense ODN; ribozyme, ribozyme ON; decoy, decoy ODN; TF, transcription factor.

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Transfection (PREVENT) I study, was the first clinical trial using genetic engineering techniques to inhibit cell-cycle activation in vein grafts (Mann *et al*. 1999). This prospective, randomized, controlled trial demonstrated the safety and biological efficacy of intra-operative transfection of human bypass vein grafts with E2F decoy oligonucleotides in a high-risk human patient population with peripheral arterial occlusion. More recently, similar results were obtained in PREVENT II, a randomized, double-blind, placebo-controlled trial investigating the safety and feasibility of E2F decoy oligonucleotides in preventing autologous vein graft failure after coronary artery bypass surgery (Dzau, 2003). Unfortunately, a recent report of a phase III trial to treat PAD patients documented no difference between E2F treatment and placebo (unpublished observation), although a phase III trial to treat ischaemic heart disease is still ongoing.

On the other hand, the transcription factor NF(B also plays a pivotal role in the coordinated transactivation of cytokine and adhesion molecule genes whose activation has been postulated to be involved in numerous diseases. It is important to note that increased NFκB binding activity has been confirmed in balloon-injured blood vessels (Yoshimura *et al*. 2001). Our recent study provided the first evidence of the feasibility of a decoy strategy against NFκB in treating restenosis (Yoshimura *et al*. 2001). Transfection of NFκB decoy ODN into balloon-injured carotid artery or porcine coronary artery markedly reduced neointimal formation (Yoshimura *et al*. 2001; Yamasaki *et al*. 2003). Based upon the therapeutic efficacy of this strategy, we treated the patients after angioplasty as a phase I/IIa trial. Overall, the decoy approach is particularly attractive for several reasons: (1) the potential drug targets (transcription factors) are plentiful and readily identifiable; (2) the synthesis of sequence-specific decoys is relatively simple and can be targeted to specific tissues; (3) knowledge of the exact molecular structure of the targeted transcription factor is unnecessary; and decoy ODN may be more effective than antisense ODN in blocking constitutively expressed factors as well as multiple transcription factors that bind to the same *cis* element. Thus, the decoy strategy may be useful for treating a broad range of human diseases. In contrast, because an important concern regarding the decoy strategy revolves around the potential inhibition of normal physiological responses, the application of the decoy strategy as gene therapy may be limited to treatment of acute conditions, namely 'transcription factor-driven diseases'.

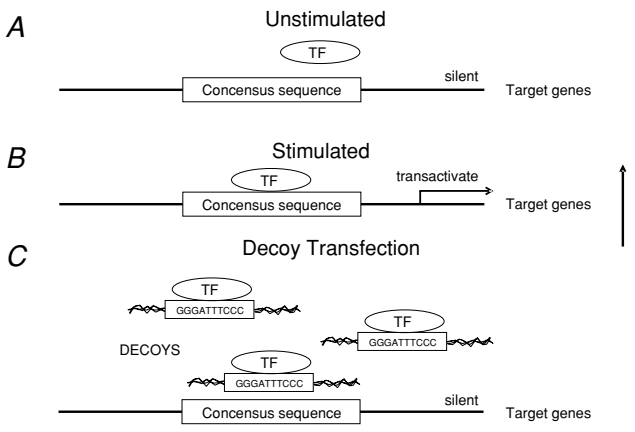

Figure 3. Scheme of 'decoy' strategy against transcription factor *A*, in the basal state, transcription factor is not bound to the *cis*-element. *B*, after stimulation, transcription factor is bound to the *cis*-element, resulting in continuous activation of target gene expression. *C*, 'decoy' *cis* element ds ODN binds to transcription factor, resulting in prevention of transactivation of transcription factor-promoting target gene expression. TF, transcription factor.

In contrast, the trials using the transfection of foreign genes are few, although inhibition of the cell cycle using non-phosphorylated retinoblastoma gene or anti-oncogenes such as *p53* and *p21* has been reported in several animal models (Chang *et al*. 1995*a*,*b*; Yonemitsu *et al*. 1998; Taniyama *et al*. 2002*b*). Recently, over-expression of inducible nitric oxide synthase gene has been tested in human subjects, although the results are not yet published. Alternatively, it has been hypothesized that rapid regeneration of endothelial cells without replication of vascular smooth muscle cells may also modulate vascular growth, because multiple antiproliferative endothelium-derived substances (e.g. NO) are secreted from endothelial cells. This concept was first tested by over-expression of *VEGF165* gene (Asahara *et al*. 1995). Based upon this finding, a human trial using *VEGF165* gene by hydrogel catheter delivery of naked VEGF165 plasmid DNA has been started for restenosis after angioplasty in peripheral artery (Isner *et al*. 1996*b*). The preliminary results documented the successful inhibition of restenosis after angioplasty (Vale *et al*. 1998). A similar trial using *VEGF165* gene has been started in Finland (Laitinen *et al*. 2000). Although gene transfer with *VEGF* using adenovirus during percutaneous transluminal coronary angioplasty (PTCA) and stenting shows that intracoronary gene transfer can be performed safely, no differences in clinical restenosis rate or minimal lumen diameter were present after the 6-month follow-up (Hedman *et al*. 2003). Further studies are necessary to prove the efficacy of re-endothelialization strategy to treat restenosis. Currently, the researchers have tried to develop gene- or decoy-eluting stents to treat restenosis.

Perspectives in gene therapy

Overall, now that gene therapy for cardiovascular disease appears to be not far from reality, it is time to take a hard look at practical issues that will determine the real clinical potential. These include: (1) further innovations in gene transfer methods (especially after the accidents using adenoviral and retroviral vectors); (2) well-defined disease targets; (3) cell-specific targeting strategies; and

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(4) effective and safe delivery systems. As gene therapy becomes a therapeutic reality, the following must be addressed: (1) safety; (2) persistence of gene expression and duration of treatment; and (3) regulation. In the future, gene transfer as a drug delivery system might overcome present limitations to identify suitable targets to treat unmet cardiovascular disease. Further modification of gene transfer methods would provide novel drug delivery system-based pharmacotherapy.

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