Plasmid-mediated gene therapy for cardiovascular disease

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Abstract  Gene transfer within the cardiovascular system was first demonstrated in 1989 yet, despite extensive basic-science and clinical research, unequivocal benefit in the clinical setting remains to be demonstrated. Potential reasons for this include the fact that recombinant viral vectors, used in the majority of clinical studies, have inherent problems with immunogenicity that are difficult to circumvent. Attention has turned therefore to plasmid vectors, which possess many advantages over viruses in terms of safety and ease of use, and many clinical studies have now been performed using non-viral technology. This review will provide an overview of clinical trials for cardiovascular disease using plasmid vectors, recent developments in plasmid delivery and design, and potential directions for this modality of gene therapy.

Keywords  Plasmids • Gene therapy • Cardiovascular disease

1. Introduction

Despite impressive therapeutic developments, cardiovascular disease is the most common cause of mortality in the developed world; an ongoing need remains for new treatments. Since the first demonstration of vascular gene transfer in 1989,1 gene therapy has been heralded as an imminent addition to the Cardiologist’s armamentarium. Cardiovascular pathologies represent the second-most popular target for clinical gene therapy. Recombinant viruses have been used for gene transfer in most clinical studies, adenovirus being employed most frequently.2 Beneficial effects of adenovirus-mediated gene transfer have been limited, however,2 due in part at least to the inflammatory responses and limited duration of transgene expression elicited by adenoviruses. The potential for serious adverse outcomes following viral vector administration has already been realized in the death of Jesse Gelsinger in a Phase 1 trial, attributed to an innate immune response to the delivered virus,3 and in the development of T-cell leukemias resulting from vector integration in proximity to proto-oncogenes in two out of 10 patients in a trial of gammaretrovirus-mediated treatment of X-linked severe combined immunodeficiency.4

Non-viral vectors offer less potential hazard than viruses and numerous preclinical studies have demonstrated successful non-viral therapeutic gene transfer to cardiovascular tissues. Several clinical trials have been completed, some showing a suggestion of clinical benefit. We will provide an overview of these clinical trials followed by a discussion of recent developments in plasmid DNA (pDNA) technology and possible future directions for plasmid-mediated cardiovascular gene therapy.

2. Plasmids as gene therapy vectors

Plasmids represent the simplest form of vector for transport of DNA into the cell nucleus. Consisting of a circular, double-stranded DNA molecule varying in size from <1000 to >200 000 bp, they are found in virtually all bacterial species where they typically encode proteins engendering antibiotic resistance. A gene therapy plasmid is represented symbolically in Figure 1. It contains a gene for antibiotic resistance regulated by a prokaryotic promoter; a prokaryotic origin of replication supporting plasmid propagation, and an expression cassette containing a promoter to initiate the transcription of the transgene encoding the therapeutic protein, the transgene itself, and a polyadenylation signal, required for the nuclear export of the mRNA. Most plasmids contain only one transgene, but polycistronic expression cassettes can encode multiple proteins and, with no size limit, plasmids may contain multiple expression cassettes.

Compared with recombinant viruses, plasmids are simple to construct and easily propagated in large quantities. They also possess an excellent safety profile, with virtually no risk of oncogenesis (as genomic integration is very inefficient) and relatively little immunogenicity. Plasmids have a very large DNA packaging capacity and can accommodate large segments of genomic DNA. They are easy to handle, remaining stable at room temperature for long periods of time (an important consideration for clinical use). The main limitation with plasmids is poor gene transfer efficiency.5 Viruses have evolved complex mechanisms to facilitate cell entry and nuclear localization. Wild-type plasmids lack these mechanisms; however, developments in delivery methods and plasmid construction may address this...
shortcoming; some such developments will be discussed later. Given
the potential benefits, plasmid-mediated gene therapy represents a
more attractive option in many respects than viral gene therapy for
cardiovascular applications.

3. Clinical studies of plasmid-mediated cardiovascular gene therapy
While several cardiovascular pathologies are potential targets for
plasmid-mediated gene therapy (Table 1), to date, all completed clini-
cal trials have addressed induction of angiogenesis.

3.1 Angiogenic gene therapy
Surgical and catheter-based interventions allow revascularization of
most patients with symptomatic coronary artery disease (CAD) or
peripheral artery disease (PAD); however, a significant proportion
of patients are not suitable for such techniques. These patients,
often with severe, end-stage disease, have few treatment options.
Therapeutic angiogenesis aims to increase perfusion of ischaemic
tissue by generation of new blood vessels. For PAD, the objective is
to relieve claudication or rest pain, hasten ischaemic ulcer healing,
and prevent amputations. Coronary angiogenesis is intended to ame-
liorate angina pectoris and perhaps improve left ventricular
contractility.

3.2 Peripheral arterial disease
The first demonstration of therapeutic angiogenesis in vivo employed
intramuscular administration of recombinant vascular endothelial
growth factor (VEGF) in rabbits. It was quickly realized that injection
of VEGF peptide produced ephemeral effects that were unlikely to
elicit clinically relevant results; subsequent investigation focused on
gene transfer. The first study of plasmid-mediated gene therapy
using pVEGF-A165 in rabbits was published in 1996. The first case
report of vascular gene transfer in humans rapidly followed: 2 µg of
pVEGF-A165 was applied to an angioplasty balloon, which was then
inflated in the popliteal artery. The patient developed leg oedema
and improved collaterals were demonstrated angiographically. Many
clinical studies of plasmid-mediated gene therapy for PAD have
been completed subsequently (Table 2). Several proangiogenic trans-
genes have been studied.

3.3 Vascular endothelial growth factor
The VEGF family contains five members: VEGF-A, VEGF-B, VEGF-C
(also known as VEGF-2), VEGF-D, and placental growth factor. VEGF-A
is the master regulator of angiogenesis: knockout of a
single allele is lethal in mice due to impaired vasculogenesis. Although

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**Table 1** Potential targets for plasmid-mediated cardiovascular gene therapy

<table>
<thead>
<tr>
<th>Clinical application</th>
<th>Potential delivery method</th>
<th>Desired onset of gene expression</th>
<th>Desired duration of gene expression</th>
<th>Possible genes</th>
<th>Target cells</th>
<th>Clinical trials initiated?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral angiogenesis</td>
<td>Intraarterial infusion; intramuscular injection</td>
<td>Not important</td>
<td>Weeks–months</td>
<td>VEGF, FGF, HGF, Del-1</td>
<td>Vessel wall (EC), myocytes</td>
<td>Phase III</td>
</tr>
<tr>
<td>Cardiac angiogenesis</td>
<td>Intracoronary infusion; myocardial injection</td>
<td>Not important</td>
<td>Weeks–months</td>
<td>VEGF, FGF, HGF</td>
<td>Vessel wall (EC), cardiomyocytes</td>
<td>Phase IIb</td>
</tr>
<tr>
<td>In-stent restenosis</td>
<td>Intracoronary infusion; bound to stent</td>
<td>Rapid</td>
<td>Weeks–months</td>
<td>VEGF, 7ND</td>
<td>Vessel wall (SMC, EC)</td>
<td>Phase II</td>
</tr>
<tr>
<td>SVG degeneration</td>
<td>Ex vivo direct application</td>
<td>Rapid</td>
<td>Months–months</td>
<td>PD-ECGF, TIMP-1, TIMP-3, fibromodulin</td>
<td>Vessel wall (SMC, EC)</td>
<td>No</td>
</tr>
<tr>
<td>Heart failure</td>
<td>Intracoronary infusion; myocardial injection</td>
<td>Not important</td>
<td>Months–permanent</td>
<td>Elastin, SERCA2a, βARKct, HGF</td>
<td>Cardiomyocytes</td>
<td>No</td>
</tr>
<tr>
<td>Bradyarrhythmias</td>
<td>Myocardial injection; coated on pacing wires</td>
<td>Rapid</td>
<td>Months–permanent</td>
<td>HCN-2, β-adrenoceptor</td>
<td>Cardiomyocytes</td>
<td>No</td>
</tr>
</tbody>
</table>

SVG, saphenous vein graft; EC, endothelial cells; SMC, smooth muscle cells.
### Table 2: Human studies of plasmid-mediated pro-angiogenic gene transfer for peripheral arterial disease

<table>
<thead>
<tr>
<th>Study name</th>
<th>Trial design</th>
<th>Number of patients</th>
<th>Promoter</th>
<th>Placebo</th>
<th>Therapeutic agent</th>
<th>Dose (at different times)</th>
<th>Delivery</th>
<th>Endpoints</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N/A</strong></td>
<td>Phase I</td>
<td>6 (7 limbs)</td>
<td>MIEhCMV</td>
<td>N/A</td>
<td>pVEGF-A165</td>
<td>2 or 4 mg im</td>
<td>Safety</td>
<td>Transient limb oedema 3/6, some evidence of increased perfusion</td>
<td>Isner et al.</td>
<td>12</td>
</tr>
<tr>
<td><strong>N/A</strong></td>
<td>Phase I</td>
<td>9</td>
<td>MIEhCMV</td>
<td>N/A</td>
<td>pVEGF-A165</td>
<td>2 × 2 mg im</td>
<td>Safety</td>
<td>Oedema 6/9, Some evidence of clinical benefit</td>
<td>Baumgartner et al.</td>
<td>11</td>
</tr>
<tr>
<td><strong>N/A</strong></td>
<td>RCT (Phase II)</td>
<td>17 (19 placebo)</td>
<td>MIEhCMV</td>
<td>Ringer’s lactate</td>
<td>pVEGF-A165-liposome</td>
<td>2 mg 10 min infusion via Dispatch or Remedy balloon catheter following balloon angioplasty</td>
<td>DSA analysis of vascularity at 3 months (1) Restenosis, Rutherford class, ABI (2)</td>
<td>Increased vascularity</td>
<td>Makenen et al.</td>
<td>13</td>
</tr>
<tr>
<td><strong>N/A</strong></td>
<td>Phase I</td>
<td>21</td>
<td>Not reported</td>
<td>N/A</td>
<td>pVEGF-A165</td>
<td>0.4-2 mg im</td>
<td>Safety and efficacy</td>
<td>Improved perfusion (assessed by MRA)</td>
<td>Shyu et al.</td>
<td>10</td>
</tr>
<tr>
<td><strong>N/A</strong></td>
<td>RCT (Phase II)</td>
<td>54 (all diabetic)</td>
<td>Not reported</td>
<td>Saline</td>
<td>pVEGF-A165</td>
<td>2 × 2 mg im</td>
<td>Amputation rate 100 days (1) Negative Secondary endpoints +ve</td>
<td>Kusumanto et al.</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td><strong>DELTA</strong></td>
<td>RCT (Phase IIa)</td>
<td>105</td>
<td>MIEhCMV</td>
<td>Poloxamer 188</td>
<td>pDel-1 and poloxamer 188</td>
<td>42 mg (11 injections) im</td>
<td>Safety at 6mo</td>
<td>Safe</td>
<td>Grossman et al.</td>
<td>17</td>
</tr>
<tr>
<td><strong>N/A</strong></td>
<td>Phase I</td>
<td>51</td>
<td>MIEhCMV</td>
<td>N/A</td>
<td>pFGF-1 (NV1FGF)</td>
<td>Multiple (0.5 – 16 mg) im</td>
<td>Safety at 6mo</td>
<td>Safe</td>
<td>Comerota et al.</td>
<td>21</td>
</tr>
<tr>
<td><strong>TALISMAN201</strong></td>
<td>RCT (Phase II)</td>
<td>125</td>
<td>MIEhCMV</td>
<td>Placebo saline injection</td>
<td>pFGF-1 (NV1FGF)</td>
<td>4 mg × 4 im</td>
<td>Ulcer healing (1) Amputation rate (2)</td>
<td>No change</td>
<td>Nikol et al.</td>
<td>22</td>
</tr>
<tr>
<td><strong>TAMARIS</strong></td>
<td>RCT (Phase III)</td>
<td>525</td>
<td>MIEhCMV</td>
<td>Placebo saline injection</td>
<td>pFGF-1 (NV1FGF)</td>
<td>4 mg × 4 im</td>
<td>Time to amputation or death (1) All amputations, death, ulcer healing, pain relief, functional status (2)</td>
<td>No change</td>
<td>Hiatt et al.</td>
<td>13</td>
</tr>
<tr>
<td><strong>N/A</strong></td>
<td>Phase I</td>
<td>6</td>
<td>MIEhCMV</td>
<td>N/A</td>
<td>pHGF</td>
<td>2 × 2 mg im</td>
<td>Safety at 2mo</td>
<td>Safe, some clinical improvement</td>
<td>Morishita et al.</td>
<td>25</td>
</tr>
<tr>
<td><strong>N/A</strong></td>
<td>Phase II/a</td>
<td>18 (22 limbs)</td>
<td>MIEhCMV</td>
<td>N/A</td>
<td>pHGF</td>
<td>2 mg × 2 or 4 mg × 2 im</td>
<td>Safety at 6mo</td>
<td>ABL, ulcer size</td>
<td>Safe, Suggestion of improvement</td>
<td>Morishita et al.</td>
</tr>
<tr>
<td><strong>HGF-stat</strong></td>
<td>RCT (Phase II)</td>
<td>104</td>
<td>MIEhCMV</td>
<td>Placebo saline injection</td>
<td>pHGF</td>
<td>0.4 mg × 3; 4 mg × 2; 4 mg × 3</td>
<td>Safety (1) Limb perfusion (TcPO2) at 6 months (1) Negative Other secondary endpoints negative</td>
<td>Ulcer healing 6 months (1) ABL, rest pain (2)</td>
<td>No change</td>
<td>Powell et al.</td>
</tr>
<tr>
<td><strong>HGF-0205</strong></td>
<td>RCT (Phase II)</td>
<td>27 (21 treatment, 6 placebo)</td>
<td>MIEhCMV</td>
<td>Placebo saline injection</td>
<td>pHGF</td>
<td>4 mg × 3 im</td>
<td>Ulcer healing at 6 months (1) ABL, rest pain (2)</td>
<td>No change</td>
<td>Improved</td>
<td>Powell et al.</td>
</tr>
<tr>
<td><strong>N/A</strong></td>
<td>RCT (Phase III)</td>
<td>40 (27 treatment, 13 placebo)</td>
<td>MIEhCMV</td>
<td>Placebo saline injection</td>
<td>pHGF</td>
<td>4 mg × 2 im</td>
<td>Ulcer size and rest pain at 3 months (1) Safety at 15 months (2)</td>
<td>Improved</td>
<td>Shigematsu et al.</td>
<td>29</td>
</tr>
</tbody>
</table>

RCT, randomized controlled trial; MIEhCMV, major immediate-early enhancer/promoter from human cytomegalovirus; ABI, ankle-brachial index; PWT, power walking test; MRA, magnetic resonance angiography; DSA, digital subtraction angiography. The numbers in parentheses in the Endpoints column refer to primary (1) and secondary (2) endpoints.
several VEGF-A isoforms exist, all studies of plasmid-mediated gene therapy for PAD have used VEGF-A165.

Several Phase I studies of intramuscular pVEGF-A165 showed that the therapy appears safe, albeit with frequent transient limb oedema (>50% in some studies), and suggested some clinical benefit.10,11 Two Phase II randomized placebo-controlled double-blind studies of pVEGF therapy for PAD have been completed subsequently. Makinen et al.12 investigated intra-arterial gene transfer at the site of angioplasty. Patients received a 10 min infusion of liposomally complexed pVEGF-A165, a VEGF-expressing adenovirus, or placebo. The therapy was well tolerated with no significant side effects. For both treatment groups, the primary endpoint of angiographically assessed vascularity was improved. Secondary endpoints, including restenosis, symptoms, and ankle-brachial index (ABI), were not improved in either group.

The second trial randomized 84 ‘no-option’ diabetic patients to intramuscular injection of pVEGF-A165 or placebo.14 The primary endpoint (amputation at 100 days) did not differ between groups. There were, however, significant improvements in VEGF-treated patients for secondary endpoints of ulcer healing, symptoms, and ABI. No major safety concerns were identified. Despite these suggestions of benefit, no Phase III trial of pVEGF therapy has been initiated at the time of writing.

3.4 Developmentally regulated endothelial cell locus-1

Developmentally regulated endothelial cell locus-1 (Del-1) is an embryonic endothelial cell protein; one of a family of proteins expressed transiently during vasculogenesis.15 Del-1 triggers angiogenesis indirectly via up-regulation of integrins αvβ5 and αvβ3.16

The DELTA-1 trial was a Phase IIa study in patients with severe PAD comparing administration of Del-1-expressing plasmid mixed with poloxamer-188 (a non-ionic copolymer) with poloxamer-188 alone.17 Both groups reported symptomatic benefit and objective improvements in leg blood flow, but there was no evidence of efficacy from pDel-1. The possibility that benefit arose from the poloxamer-188 carrier was investigated subsequently using a saline-only control group. This trial reported negative results in 2006: (http://www.medicalnewstoday.com/articles/47007.php).

3.5 Fibroblast growth factor

The fibroblast growth factor (FGF) family comprises 23 members. FGF-1 (also known as acidic FGF: aFGF) and FGF-2 (also known as basic FGF: bFGF) are the prototypic members and, although their biology remains incompletely understood, they elicit angiogenesis following virus-mediated gene transfer in vitro and in vivo.18,19

The plasmid used in clinical studies of FGF therapy (NV1FGF) is unconventional, consisting of an expression cassette inserted into a plasmid backbone devoid of antibiotic resistance genes (designated pCOR) that can only be propagated in a special Escherichia coli strain.20 This offers a smaller bacterially derived backbone than most plasmids, thereby reducing the quantity of potentially immunogenic bacterial DNA.

A Phase I dose-finding study of intramuscular NV1FGF in patients with ‘no-option’ PAD revealed no safety concerns and a suggestion of improvement in symptoms and some markers of perfusion.21 NV1FGF was investigated further in the Phase II TALISMAN201 study: 125 patients unsuitable for revascularization, with PAD and non-healing leg ulcers were randomized to NV1FGF (16 mg in divided injections) or saline.22 Although there was no improvement in ulcer healing (the primary endpoint), there were significant improvements in some secondary endpoints including a >50% reduction in amputations.

The TALISMAN201 results provided the basis for the Phase III TAMARIS trial which reported at the AHA Scientific Sessions in 2010.23 TAMARIS randomized 525 patients to treatment with four 4 mg doses of NV1FGF or placebo. The primary endpoint was major amputation or death at 12 months; secondary endpoints were all amputations, death, ulcer healing, pain relief, and functional status. This well-conducted trial was conclusively negative with no effect on any endpoints. Although no safety concerns were identified, the results render any future development of NV1FGF unlikely.

3.6 Hepatocyte growth factor

Hepatocyte growth factor (HGF) is a potent angiogenic and antifibrotic protein. It induces collateral formation in ischaemic rabbit hindlimbs24,25 and is down-regulated in the limb vessels of PAD patients. An HGF-expressing vector (Collategene™, Anges MG Inc.) has been investigated in a series of trials in Japan and the USA.

Two Phase I/IIa studies showed that intramuscular pHGF appeared safe in ‘no-option’ PAD patients and suggested some clinical improvement.26,27 The Phase II HGF-STAT trial randomized 104 patients with rest pain or ischaemic ulcers to three doses of pHGF (multiple injections totalling 1.2, 8, or 12 mg) or placebo.28 Limb perfusion (assessed by transcutaneous oxygen tension: TcPO2) at 6 months was increased in the high-dose group compared with all other groups. However, other secondary endpoints (including ABI, amputation, and wound healing) were not improved. No difference was reported in side effects or complications between groups. In a follow-on Phase II study, 21 patients received a total dose of 12 mg of intramuscular pHGF and six patients received placebo injections.28 Whilst all patients in HGF-STAT received injections in the same location in the lower limbs, in this second study, the site of injection was at the site of the subjects’ ischaemia as assessed angiographically. There was no difference in the primary endpoint of ulcer healing at 6 months, but the study was underpowered because enrolment was stopped with only half the predicted number of subjects randomized. However, the secondary endpoints of rest pain and ABI were improved in the treatment group, with no increase in adverse events.

A Japanese Phase III study randomized 40 patients to treatment with a total of 8 mg pHGF or placebo.29 At 12-week follow-up, the primary endpoint of rest pain or ischaemic ulcer size was significantly improved in the treatment group. No adverse effects were identified. These results have prompted the patent holders to initiate a global Phase III study, aiming to recruit 560 no-option/poor-option PAD patients. The development of Collategene™ has been granted Fast-Track status by the FDA.

3.7 Coronary artery disease

Clinical experience of plasmid-mediated angiogenic therapy for CAD is confined to studies of VEGF. In the first study, five no-option patients with severe angina and multivessel CAD received 125 µg of pVEGF-A165 by injection into the anterolateral left ventricular myocardium at mini-thoracotomy.20 There were no safety issues and all patients derived symptomatic benefit with some improvements in objective measures of ischaemia. This experience was expanded upon with another 15 patients (the last 10 receiving 250 µg of
plasmid) with equally promising results. Similar findings were subsequently reported by other groups using pVEGF-A$_{165}$ or pVEGF-C.$^{32-35}$ The problems with these studies were two-fold: it was difficult to accurately identify the areas of ischaemic myocardium to be treated, and an invasive procedure (i.e. a thoracotomy) may have a large placebo effect, but it was considered unethical to include a placebo surgery arm. The first problem led to development of the NOGA system (Figure 2), which has been used in most recent clinical trials of myocardial angiogenic gene therapy.$^{36-41}$ A sensor-tipped catheter is placed percutaneously into the left ventricle. A real-time three dimensional electromechanical map of the heart is created by determining the location of the catheter using a magnetic field (allowing measurement of contractility) whilst simultaneously measuring endocardial electrical signals (which correlate with ischaemia).$^{41}$ This technique identifies areas of viable myocardium as accurately as stress echocardiography and SPECT$^{42}$ and was originally used to guide myocardial injections at thoracotomy.$^{41}$ The development of percutaneous intramyocardial injection catheters like the MyoStar (Figure 3) has made possible the design of ethical placebo-controlled trials.

Three Phase II studies have used the NOGA system and a percutaneous injection catheter to deliver pVEGF.$^{38,40}$ The Euroinject One study randomized 80 no-option patients to intramyocardial injection of 500 µg pVEGF-A$_{165}$ or placebo plasmid.$^{38}$ At 3 months, no effect was detected on the primary endpoint of myocardial stress perfusion defects. There was, however, a suggestion of improved myocardial contractility in the treatment group. The NORTHERN study randomized 93 patients to intramyocardial pVEGF-A$_{165}$ or saline.$^{40}$ Despite using a higher pVEGF-A$_{165}$ dose (2000 µg) than Euroinject One, no effect was observed on the primary endpoint of change in myocardial perfusion. There were significant, but similar, improvements in myocardial perfusion, exercise treadmill time, and angina in both treatment and placebo groups. The GENASIS study randomized patients to pVEGF-C or placebo delivered via the Stiletto catheter (Boston Scientific, MA, USA) (http://clinicaltrials.gov/ct2/show/NCT00090714). This study was terminated early, with only 295 of a planned 404 patients enrolled, after interim analysis showed that the primary endpoint of improved exercise treadmill time at 3 months follow-up was unlikely to be reached. Preliminary results were reported in 2006 (http://www.medicalnewstoday.com/articles/53786.php) but remain unpublished.

Bone marrow-derived haematopoietic stem cells have the potential to enhance angiogenesis. A Phase I study assessed treatment with 6 days of subcutaneous granulocyte colony-stimulating factor (GCSF) administered 1 week after NOGA-guided intramyocardial pVEGF-A$_{165}$ injection.$^{39}$ Recipients were compared with a control group of patients previously enrolled in the Euroinject One trial. Despite a

Figure 2 (A) NOGA endomyocardial electromagnetic mapping catheter (Cordis, NJ, USA). (B) Tip detail.

Figure 3 (A) The MyoStar catheter (Cordis) combines endomyocardial (NOGA) mapping capability with the ability to deliver intramyocardial injections. (B) Tip detail showing the extended injection needle.
10-fold increase in circulating CD34+ stem cells, no improvement in myocardial perfusion was seen by comparison with groups receiving no GCSF.

Multiple transgenes may enhance angiogenesis to a greater extent than a single transgene. The VIF-CAD study is a randomized double-blind trial comparing the effects of MyoStar-mediated intramyocardial injection of placebo plasmid with a bicistronic plasmid expressing FGF-2 and VEGF-A165 (http://clinicaltrials.gov/ct2/show/NCT00620217). The primary endpoint is change in SPECT-assessed myocardial perfusion at 4 months. Enrolment is completed, but results are currently unreported.

3.8 In-stent restenosis

Percutaneous coronary intervention is the dominant form of revascularization for CAD. Despite developments in stent technology, restenosis remains a significant problem. The KAT trial is currently the only published clinical study of gene therapy for coronary restenosis. Initial balloon angioplasty was followed by immediate delivery using a Dispatch per fusion–infusion catheter (Figure 4) of placebo, VEGF-A165-expressing adenovirus, or plasmid/liposome complexes expressing VEGF-A165. Following delivery, stents were deployed in 90% of patients. At 6 months, angiographic restenosis was unaffected in either treatment group.

4. Why so little benefit from therapeutic gene transfer?

Results of clinical trials of plasmid-mediated cardiovascular gene therapy to date have been disappointing. So, are the transgenes studied simply ineffective for the use to which they have been put? Given the positive outcomes in animal models, it is likely that subtherapeutic transgene expression remains a major problem in the clinical setting, with inadequate protein production for insufficient lengths of time to produce a useful effect. Some factors influencing the effects of angiogenic cytokines may not be reproducible in preclinical models, however: under conditions of oxidative stress, as occurring in atherosclerotic arteries, VEGF does not enhance angiogenesis whereas HGF does. This may explain the encouraging results in HGF trials and the relative lack of effect of VEGF, despite the preclinical efficacy of both transgenes. It is difficult to quantify levels of local transgene expression in humans; trials have relied on empirically determined doses derived from preclinical studies. Perhaps, all that is needed to achieve clinical benefit is to use higher doses of plasmid. However, 2 mg of plasmid was ineffective in the NORTHERN study and a total of 16 mg of NV1FGF elicited no therapeutic effect in the TAMARIS trial. It is likely that strategies to facilitate target-cell plasmid uptake will have to be combined with modifications of the plasmids employed (to enhance magnitude and duration of transgene expression) in order to achieve therapeutic efficacy.

5. Improving plasmid-mediated gene transfer

Inefficient gene transfer is the Achilles heel of plasmid-mediated gene therapy. This is a consequence of several barriers existing between vector introduction and therapeutic protein production.

- When introduced systemically, pDNA is degraded by serum nucleases and eliminated by the liver.
- Target organs for cardiovascular gene therapy possess a relatively impermeable, continuous, vascular endothelium.
- Plasmids do not have mechanisms to facilitate crossing of cell membranes.
- After cellular entry, plasmids must avoid lysosomal and cytoplasmic nucleases before localizing to the nucleus.
- Entry to the nucleus, through nuclear pore complexes, occurs slowly and inefficiently in non-mitotic cells.
- Plasmids may persist extrachromosomally in quiescent cells but are rapidly lost from dividing cells. When episcopal persistence occurs, plasmids are susceptible to transcriptional silencing with limited longevity of transgene expression.

Potential exists to improve gene transfer efficacy at each of these barriers: physical techniques and carrier vehicles can aid target-cell localization and intracellular entry, while avoiding lysosomal/cytosolic degradation; nuclear localization signals can aid intracellular trafficking; promoter and enhancer optimization can increase target-cell gene expression whilst reducing expression in non-target organs.

5.1 Delivery mechanisms within the cardiovascular system

Direct delivery to target organs is the simplest means of avoiding the pitfalls of systemic pDNA injection. Leg skeletal muscle is readily amenable to injection, and this method has been applied in most trials of angiogenic therapy for PAD. Intramyocardial injection using percutaneous catheter-based systems (discussed above) offers an equivalent approach to the cardiac muscle and has been reviewed recently. Other routes for myocardial delivery include retropericardial injection and intracoronary infusion. Selective retrofusion of virus into the cardiac veins achieved more uniform myocardial transduction in large animals than direct intramyocardial injection, but has not been applied to non-viral gene transfer.

Coronary stents represent an ideal platform for arterial gene transfer, remaining in place permanently and allowing sustained local exposure to gene transfer vectors. This is discussed later.
5.2 Physical methods to enhance cellular uptake

Electroporation (application of a high-voltage electrical pulse to cells) increases transfection in vitro by causing temporary pore formation in cell membranes.\(^5\) Electroporation improves gene transfer in the skeletal muscle and myocardium in vivo,\(^5\) but may cause significant tissue damage\(^5\) and required a thoracotomy for myocardial application, where it was also associated with ventricular arrhythmias.\(^5\)

Electroporation can be applied to vessels in vivo by surgical exposure and adventitial electrode application,\(^4\) but this may be too invasive for clinical use. Electroporation-enhanced transfection of porcine arteries was demonstrated using a modified balloon catheter\(^5\) and electroporation-mediated plasmid transfer into vein grafts \(\text{in vivo}\) achieved potentially therapeutic effects.\(^5\) Electroporation remains to be investigated in human trials. Before such application, concerns about the safety of high voltages applied to coronaries or myocardium need to be assessed. Application to vein graft conduits \(\text{in vivo}\) represents the most likely initial avenue for clinical exploitation of electroporation-enhanced gene transfer.

Ultrasound-targeted destruction, at the target site, of plasmid-bearing cationic microbubbles enhances the efficacy of cardiovascular gene transfer\(^3\) and has been applied preclinically.\(^5\) The non-invasive nature and localized delivery resulting from this technique are major attractions for clinical use. Other physical techniques to improve gene transfer, including the gene gun and laser irradiation, are less suited to the cardiovascular system.

5.3 Carrier vehicles for plasmid DNA

Carrier vehicles for pDNA should reduce susceptibility to circulating nucleases and increase cellular uptake. They may also target plasmids to a specific tissue. Like carrier microbubbles, most vehicles are cationic. Their positive charge enables electrostatic complex formation with negatively charged pDNA. Complexes are prepared with a residual positive charge which enhances cellular uptake via electrostatic interaction with the negatively charged cell membrane. These carrier vehicles can substantially increase transfection; non-viral systems can be as effective as viruses at delivering DNA to cell nuclei.\(^6\)

The efficacy of cationic liposomes varies with the cell-type studied and is typically poor in vascular wall cells.\(^6\) Non-specific delivery is a problem with unmodified liposomes; intravenous administration of complexes can elicit widespread systemic transfection, particularly within the lung.\(^6\) and uncomplexed liposomes can be cytotoxic.\(^6\) Nonetheless, liposome–plasmid complexes have been used in several clinical trials with no safety issues (Tables 2 and 3). It is possible to target liposomes by inclusion of cell-specific ligand-targeting peptides within the complex,\(^6\) eliciting enhanced gene transfer and efficient transduction on clinically relevant timescales. Such receptor-targeted nanocomplexes have not been studied clinically.

Cationic non-liposomal polymers improve plasmid-mediated gene transfer in the skeletal muscle,\(^6\) and polyamidoamine dendrimers improved arterial gene delivery using an adventitial collar.\(^6\) The only clinical experience of cationic polymers is with poloxamer-188, discussed earlier.\(^5\) Negatively charged poloxamine nanospheres are potentially less toxic than cationic carriers and have significantly enhanced mouse myocardial transfection.\(^7\)

5.4 Plasmid modification

Almost all clinical studies have used basic plasmids for gene transfer, with simple transgene expression cassettes and substantial bacterial elements. Although there is an argument for using small plasmids (plasmid size is inversely related to transfection efficiency\(^7\)) manipulation of the plasmid backbone can improve transgene expression and target-cell specificity with relatively little increase in plasmid size. Components amenable to modification include the promoter of transgene expression, other enhancer elements, nuclear localization signals, prokaryotic components, and CpG-dinucleotide content.

The most commonly used promoter in clinical cardiovascular gene therapy is the major intermediate-early enhancer/promoter from human cytomegalovirus (MIEhCMV). This powerful constitutively active promoter is commonly used in vectors designed for vascular gene therapy.\(^7\) Its continued widespread use is surprising, however; the equivalent promoter from murine cytomegalovirus (MIEmCMV) was shown in 1997 to elicit greater expression than MIEhCMV in all cell lines tested,\(^7\) and a truncated MIEmCMV has even greater activity.\(^7\) Despite the markedly higher levels of expression achieved, MIEmCMV remains to be used in clinical studies.

Promoters of viral origin are prone to transcriptional silencing, which limits duration of transgene expression.\(^7\) This problem has been addressed preclinically using cell- or tissue-specific mammalian promoters. A range of promoters exists that confer specificity of expression to smooth muscle cells (SMC),\(^7\) endothelial cells (EC),\(^7\) cardiomyocytes,\(^7\) ischaemic myocardium,\(^7\) or ‘vascular tissue’ (SMC and EC).\(^7\) However, none achieve greater transgene expression than MIEhCMV in their respective cells/tissues,\(^7\) and there is no evidence of improved duration of expression. The potential advantages of these promoters remain theoretical.

Other plasmid modifications have been studied.

- Several different introns inserted between the promoter and transgene increase gene expression.\(^7\)
- Several enhancer sequences markedly increase expression from SMC-specific and ubiquitously active viral promoters in SMC.\(^7\) Structural and regulatory chromosomal elements can increase magnitude and duration of transgene expression in non-vascular tissues.\(^7\)
- Inclusion of DNA nuclear-targeting sequences increases transgene expression up to 40-fold.\(^7\)
- Inclusion of the gene for the Epstein–Barr nuclear antigen-1 and its binding site oriP (both from the Epstein–Barr virus) can increase transgene expression up to 100-fold.\(^7\)

Although far less immunogenic than viruses, the presence within plasmids of unmethylated CpG dinucleotides can induce innate immune system activation and host inflammatory responses.\(^7\) Completely CpG-free plasmids reduce inflammation and increase gene expression;\(^7\) however, complete elimination of CpG dinucleotides dramatically limits the choice of promoter and enhancer elements, which may outweigh any benefits from reducing host immune/inflammatory responses.

DNA minicircles are derived from plasmid DNA, but consist solely of an expression cassette, lacking any bacterial components. They increase transduction efficiency both in vitro and in vivo.\(^7\) They are more complex to produce than standard plasmids and have not been used in clinical studies of cardiovascular gene therapy.
Table 3 Human studies of plasmid-mediated pro-angiogenic gene transfer for CAD

<table>
<thead>
<tr>
<th>Study name</th>
<th>Trial design</th>
<th>Number of patients</th>
<th>Promoter</th>
<th>Placebo</th>
<th>Therapeutic agent</th>
<th>Dose</th>
<th>Delivery</th>
<th>Endpoints</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/A</td>
<td>Phase I</td>
<td>20</td>
<td>MIEhCMV</td>
<td>N/A</td>
<td>pVEGF-A&lt;sub&gt;165&lt;/sub&gt;</td>
<td>125–250 µg</td>
<td>Intramyocardial injection (mini-thoracotomy)</td>
<td>Myocardial perfusion (SPECT)</td>
<td>Improved</td>
<td>Losordo et al.¹⁰; Symes et al.¹¹</td>
</tr>
<tr>
<td>N/A</td>
<td>Phase I</td>
<td>13</td>
<td>MIEhCMV</td>
<td>N/A</td>
<td>pVEGF-A&lt;sub&gt;165&lt;/sub&gt;</td>
<td>250–500 µg</td>
<td>NOGA mapping; Intramyocardial injection (mini-thoracotomy)</td>
<td>Myocardial perfusion (SPECT) and area of NOGA ischaemia (not primary)</td>
<td>Improved</td>
<td>Vale et al.¹¹</td>
</tr>
<tr>
<td>N/A</td>
<td>Phase I</td>
<td>13</td>
<td>MIEhCMV</td>
<td>placZ or Ringer's lactate</td>
<td>pVEGF (as liposome complex; mouse VEGF)</td>
<td>1000 µg</td>
<td>Intracoronary perfusion-infusion Dispatch catheter following PCI</td>
<td>Restenosis at 6 months</td>
<td>Negative</td>
<td>Laitinen et al.¹²²</td>
</tr>
<tr>
<td>N/A</td>
<td>RCT (Phase I)</td>
<td>6</td>
<td>Not stated</td>
<td>Sham procedure (no injection)</td>
<td>pVEGF-C (VEGF-2)</td>
<td>200 µg</td>
<td>Percutaneous intramyocardial (NOGA and MyoStar catheter)</td>
<td>Symptoms</td>
<td>Myocardial perfusion</td>
<td>Improved</td>
</tr>
<tr>
<td>N/A</td>
<td>Phase I/II</td>
<td>7</td>
<td>MIEhCMV</td>
<td>N/A</td>
<td>pVEGF-A&lt;sub&gt;165&lt;/sub&gt;</td>
<td>250–1000 µg</td>
<td>Intramyocardial injection (mini-thoracotomy)</td>
<td>Myocardial perfusion</td>
<td>Improved</td>
<td>Sylven et al.¹²²; Sarkar et al.¹³</td>
</tr>
<tr>
<td>N/A</td>
<td>RCT (Phase I/II)</td>
<td>19</td>
<td>Not stated</td>
<td>Saline</td>
<td>pVEGF-C (VEGF-2)</td>
<td>200–2000 µg</td>
<td>Percutaneous intramyocardial (NOGA and MyoStar catheter)</td>
<td>CCS angina class at 4 months (1)</td>
<td>Positive</td>
<td>Losordo et al. 2002¹⁷</td>
</tr>
<tr>
<td>N/A</td>
<td>Phase I</td>
<td>30</td>
<td>Not stated</td>
<td>N/A</td>
<td>pVEGF-C (VEGF-2)</td>
<td>200, 800, or 2000 µg</td>
<td>Intramyocardial injection (mini-thoracotomy)</td>
<td>Symptoms</td>
<td>Improved</td>
<td>Reilly et al.³⁴; Fortuin et al.³⁵</td>
</tr>
<tr>
<td>KAT</td>
<td>RCT (Phase II)</td>
<td>103</td>
<td>MIEhCMV</td>
<td>Ringer’s lactate</td>
<td>pVEGF-A&lt;sub&gt;165&lt;/sub&gt; (as liposome complex)</td>
<td>2000 µg</td>
<td>Intracoronary infusion-perfusion Dispatch catheter</td>
<td>Restenosis at 6 months (1)</td>
<td>Negative</td>
<td>Hedman et al.⁴³</td>
</tr>
<tr>
<td>Euroinject One</td>
<td>RCT (Phase II)</td>
<td>80</td>
<td>MIEhCMV</td>
<td>Placebo plasmid</td>
<td>pVEGF-A&lt;sub&gt;165&lt;/sub&gt;</td>
<td>500 µg</td>
<td>Percutaneous intramyocardial (NOGA and MyoStar catheter)</td>
<td>Myocardial perfusion at 3 months (SPECT) (1)</td>
<td>Negative</td>
<td>Kastrup et al.³⁸</td>
</tr>
<tr>
<td>N/A</td>
<td>Phase I</td>
<td>16</td>
<td>MIEhCMV</td>
<td>Control group from EUROINJECT ONE</td>
<td>pVEGF-A&lt;sub&gt;165&lt;/sub&gt; followed by GCSF</td>
<td>500 µg</td>
<td>Percutaneous intramyocardial (NOGA and MyoStar catheter)</td>
<td>Myocardial perfusion at 3 months (SPECT) (1)</td>
<td>Negative</td>
<td>Ripa et al.³⁹</td>
</tr>
<tr>
<td>NORTHERN</td>
<td>RCT (Phase I/III)</td>
<td>93</td>
<td>MIEhCMV</td>
<td>Buffered saline</td>
<td>pVEGF-A&lt;sub&gt;165&lt;/sub&gt;</td>
<td>2000 µg</td>
<td>Percutaneous intramyocardial (NOGA and MyoStar catheter)</td>
<td>Myocardial perfusion at 3 or 6 months (SPECT) (1)</td>
<td>Negative</td>
<td>Stewart et al.⁴⁰</td>
</tr>
<tr>
<td>VIF-CAD</td>
<td>RCT (Phase II)</td>
<td>52 planned</td>
<td>MIEhCMV</td>
<td>Placebo plasmid</td>
<td>pFGF-2/VEGF-A&lt;sub&gt;165&lt;/sub&gt; (bicistronic)</td>
<td>500 µg</td>
<td>Percutaneous intramyocardial (MyoStar catheter)</td>
<td>Myocardial perfusion at 4 months (SPECT) (1)</td>
<td>Unpublished</td>
<td>Unpublished</td>
</tr>
<tr>
<td>GENASIS</td>
<td>RCT (Phase IIb)</td>
<td>295</td>
<td>Not stated</td>
<td>Not stated</td>
<td>pVEGF-C (VEGF-2)</td>
<td>Not stated</td>
<td>Percutaneous intramyocardial (Siletto catheter)</td>
<td>Improvement in ETT at 3 months (1)</td>
<td>Negative at interim analysis (stopped early 2006)</td>
<td>Unpublished</td>
</tr>
</tbody>
</table>

RCT, randomized controlled trial; GCSF, granulocyte colony-stimulating factor; SPECT, single positron emission tomography; MIEhCMV, major immediate-early enhancer/promoter from human cytomegalovirus; ETT, exercise tolerance test; PCI, percutaneous coronary intervention.

The numbers in parentheses in the Endpoints column refer to primary (1) and secondary (2) endpoints.

R.D. Williams et al. ⁵⁷²
6. What is the future for plasmid-mediated cardiovascular gene therapy?

It is difficult not to be disappointed by the results of clinical cardiovascular non-viral gene therapy so far, with only pHGF (Collategene™) still undergoing active investigation in Phase III clinical trials. Despite this, the advantages of virus-free gene transfer are still sufficiently attractive to continue to nurture the belief, voiced in 2002, that "with the development of new techniques to increase non-viral expression levels, plasmids will be the vector of choice for human applications."^107^ A variety of approaches exists to improve the ability of plasmid-mediated gene transfer to elicit therapeutic protein expression in cardiovascular tissues, yet at the time of writing, almost none have been applied clinically. It is imperative that these technical issues are remedied in the next generation of clinical studies; repeated failure in trials of clinical gene transfer is unlikely to foster long-term investment in such therapies.

All studies, to date, of cardiovascular non-viral gene therapy have addressed angiogenesis. This represents an obvious target, with a population of patients for whom no alternative therapies exist and where short-term gene expression might offer a long-term therapeutic effect. There are, however, a range of pathologies that might be targeted once non-viral gene therapy achieves some clinical effectiveness. The efficacy of local drug delivery from coronary stents is well established, but drug-eluting stents are associated with late complications related to delayed healing. Plasmid-eluting stents offer the possibility of avoiding cytostatic/cytotoxic agents: the concept of stent-based plasmid delivery to the vasculature has been proven in several preclinical studies (Table 4).^108 – 113^ Prevention of saphenous vein aorto-coronary bypass conduit disease is a tempting target for gene therapy too, as vein segments are conveniently ex vivo during surgical preparation. Several preclinical studies have demonstrated reduced vein graft neointima following plasmid-mediated gene transfer.^114,115^ Myocardial gene therapy has recently attracted interest following the initiation of clinical studies using adeno-associated virus to treat heart failure (recent reviews discuss this in detail^116,117^). The CUPID study of SERCA2a (sarcoplasmic reticulum calcium ATPase) gene transfer^118^ reported improved symptoms, functional status, and ventricular volumes 6-month post-treatment at EuroHF 2010. Two preclinical studies have shown beneficial effects following HGF-expressing plasmid delivery in models of severe left ventricular dysfunction, following intramyocardial injection^119^ and electroporation-facilitated injection into the skeletal muscle.120

Finally, biopacemaking offers a means of augmenting (and eventually replacing) the function of permanent pacemakers for sinoatrial or atrioventricular node disease. A recent preclinical study demonstrated efficacy following intramyocardial injection of plasmids expressing two transgenes (the hyperpolarization-activated HCN2 channel and the β2-adrenergic receptor).^121^ Initial clinical studies of such therapies will require concomitant permanent pacemaker implantation. The intriguing option presents itself of coating the pacing wire tips with a vector to allow localized myocardial delivery. Gene therapy for hypertension and hypercholesterolaemia has been studied preclinically too, but the target tissues are typically non-cardiovascular.

### Table 4 Preclinical studies of plasmid-coated gene-eluting stents

<table>
<thead>
<tr>
<th>Transgenes</th>
<th>Model</th>
<th>Stent coating</th>
<th>Method of vector attachment</th>
<th>Dose/stent</th>
<th>Primary outcome</th>
<th>Change in outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>Porcine coronary</td>
<td>PLGA polymer</td>
<td>Dipcoated in polymer–plasmid mix</td>
<td>900–1100 µg</td>
<td>Transfection Success (1% transfection)</td>
<td>Klugherz et al.</td>
<td></td>
</tr>
<tr>
<td>GFP</td>
<td>Porcine coronary</td>
<td>PLGA polymer</td>
<td>Dipcoated in polymer–plasmid mix</td>
<td>900–1100 µg</td>
<td>Transfection Success (1% transfection)</td>
<td>Klugherz et al.</td>
<td></td>
</tr>
<tr>
<td>Luc, GFP</td>
<td>Rabbit iliac</td>
<td>Denatured collagen and PLGA polymer</td>
<td>Incubated in polymer–plasmid mix</td>
<td>500 µg</td>
<td>Not reported (performed by biocompatibles)</td>
<td>Walter et al.</td>
<td></td>
</tr>
<tr>
<td>NOS</td>
<td>Rabbit carotid</td>
<td>Anti-DNA antibody-collagen coated stent</td>
<td>Incubated in plasmid solution with lipofectin</td>
<td>Not reported</td>
<td>Neointima formation Reduction</td>
<td>Walters et al.</td>
<td></td>
</tr>
<tr>
<td>GFP</td>
<td>Rabbit carotid</td>
<td>Anti-DNA antibody-collagen coated stent</td>
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</tr>
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<tr>
<td>GFP</td>
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<td>Anti-DNA antibody-collagen coated stent</td>
<td>Incubated in plasmid solution with lipofectin</td>
<td>Not reported</td>
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<td></td>
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<tr>
<td>GFP</td>
<td>Rabbit carotid</td>
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<tr>
<td>GFP</td>
<td>Rabbit carotid</td>
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<td>Walters et al.</td>
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<td>Walters et al.</td>
<td></td>
</tr>
</tbody>
</table>
Sequencing of the human genome has left the doctors of our generation with a horde of potentially therapeutic DNA sequences that might be applied to human pathologies. It is almost unimaginable that transfer of nucleic acids for therapeutic purposes will not someday be part of the standard armamentarium of the hospital practitioner, but there is an inescapable need for progress beyond the meagre achievements outlined so far within the cardiovascular system. This progress must be in the technical areas of optimization of gene delivery and expression, which requires determination of the best modality of gene transfer (viral or non-viral), as well as establishing the most effective gene for each clinical purpose. Although the promise of large-scale clinical trials is more than embryonic and the results of the planned Phase III trial of plasmid-mediated delivery of HGF for PAD are eagerly awaited, there are many hurdles to cross before gene therapy in the vasculature becomes a daily clinical reality.

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