WILEY
Interdisciplinary Reviews
NANOMEDICINE

Article type: Advanced Review

Non-viral gene therapy for myocardial engineering

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Keywords

Gene therapy, Myocardial infarction, Non-viral, Cell-based therapy, Angiogenesis

Abstract

Despite significant advances in surgical and pharmacological techniques, myocardial infarction remains the main cause of morbidity in the developed world because no remedy has been found for the regeneration of infarcted myocardium. Once the blood supply to the area in question is interrupted, the inflammatory cascade, among other mechanisms, results in the damaged tissue becoming a scar. The goals of cardiac gene therapy are essentially to minimize damage, to promote regeneration or some combination thereof. While the vector is, in theory, less important than the gene being delivered, the choice of vector can have a significant impact. Viral therapies can have very high transfection efficiencies, but disadvantages include immunogenicity, retroviral-mediated insertional mutagenesis and the expense and difficulty of manufacture. For these reasons, researchers have focused on non-viral gene therapy as an alternative. In this review, naked plasmid delivery, or the delivery of complexed plasmids, and cell-mediated gene delivery to the myocardium will be reviewed. Pre-clinical and clinical trials in the cardiac tissue will form the core of the discussion. While unmodified stem cells are sometimes considered therapeutic vectors based on paracrine mechanisms of action basic understanding is limited. Thus, only genetically modified cells will be discussed as cell-mediated gene therapy.

Ischemic heart disease, which includes acute damage due to myocardial infarction (MI) and chronic damage due to atherosclerotic narrowing of vessels, accounts for 35% of deaths reported in the United States every year [1]. MI, which is literally the death of cardiac tissue due to lack of

oxygen supply, is the result of the occlusion of a coronary artery. Within a few hours of interrupted blood supply, affected cardiomyocytes die. While the body has adaptive mechanisms, such as hypertrophy of the undamaged cardiac muscle and the development of collateral blood supply to minimize the damage, ischemic heart disease remains the most common cause of death in developed countries [2].

The delivery of plasmid DNA to the myocardium is a complicated problem as the transfection efficiency of unmodified DNA is quite low, but complexation with agents designed to improve transfection can increase the cytotoxicity of the treatment [3]. Delivery of uncomplexed plasmid DNA is conceptually the simplest gene delivery technique, but these plasmids have extremely low transfection efficiencies *in vitro* and are not particularly effective *in vivo.* Compared to viral vectors, transgene expression is almost negligible and the expression time is also very limited [4]. However, there are significant advantages in using plasmids instead of viruses, such as ease of preparation (large and small scale), very low immunogenicity (as there are essentially no protein or membrane components in the plasmid), capacity for long term storage, ease of recombinant manipulation and much larger expression cassettes [4]. A variety of non-viral techniques are available to improve transfection efficiency of plasmids. These include liposomes, a variety of linear, branched, hyper-branched and dendrimeric polymers and nanoparticles [5]. The complexes must have an overall positive charge, as the route of internalisation requires interactions with the negatively charged phospholipid membrane of the cell [6].

The choice of gene partly depends on which of a wide variety of cardiac problems is being addressed. A relatively small but exciting area of study is concerned with the pace-making activity of the heart. The goal of these therapies is to reduce the risk of death due to fibrillation or arrhythmias [7]. However, the majority of research into gene therapy for heart disease ultimately aims to minimize or reverse the ischemic damage that occurs as a result of MI. Preventing or decreasing ischemia/reperfusion induced cell-death, attenuating the inflammatory reaction and reducing adverse remodelling of the myocardium are all effective strategies for decreasing the loss of function post-MI. Stimulating angiogenesis is another extensively investigated area, with more than 25 clinical trials using non-viral vectors. Angiogenesis is particularly important for hypertrophied myocardium, as the metabolic demands are significantly higher than normal myocardium but the capillary density is unchanged [8].

The actual therapeutic administration of the gene therapy treatment is an often overlooked area, but it is of extreme importance. Different administration techniques have significantly different efficiencies and may deliver the genes to more or less clinically relevant sites. They may also determine the level of risk for the patient undergoing the procedure.

Genetic engineering of cardiac tissue has been demonstrated to be both feasible and beneficial in preclinical studies, although significant optimisation is still required. Selection of the vector, gene and administration route can determine the effectiveness of the overall treatment as well as side-effects. This review summarizes the work done with non-viral vectors and will focus on the available vectors, therapeutic gene and administration techniques, and including a brief discussion of clinical trials in the area.

Vectors for Gene Delivery

Significance

The manner in which a gene is delivered is far more important than that may be immediately obvious. The vector can have a significant influence on the therapeutic efficacy and costs. In addition, serious side effects including death can occur.

Viral therapies, in general, have significantly higher transfection efficiencies than non-viral therapies. This is not surprising, considering that viruses have evolved to overcome the natural protective mechanisms cells have developed to destroy any unprotected genetic material that enters the cytoplasm.

Regulatory hurdles are significantly greater for viral therapies compared to non-viral therapies. There have been a few deaths directly linked to viral therapies [9], but these fatalities have highlighted the potential risks. Viral therapies also require more safety regulations in preparation, requiring specialized laboratories and careful handling. Non-viral therapies, conversely, have demonstrated negligible safety risks and can be prepared with relative ease. These considerations also translate into expense. Viral vectors, which require more specialized facilities, more highly trained personnel, and have strict requirements for shipping and handling, are therefore significantly more expensive than their non-viral counterparts, which can be mass-produced, are reasonably stable at atmospheric conditions, and are relatively easy to handle. Furthermore, as there is less risk of negative side effects, the monitoring of the patient is less important, and the actual treatment procedures are more straightforward.

Vector

The highest transfection efficiencies are obtained with viral vectors. However, non-viral transfection reagents have begun to address the problem of the extremely low transfection efficiencies observed with naked plasmids. Gebhart *et al.* have reported on a relatively thorough comparison of a number of non-viral vectors in a variety of cell lines in terms of luciferase activity [10], and Uchida *et al.* present a similar comparison in terms of percentage LacZ positive cells [11]. Efficiencies as high as 35% have been observed with polymer based non-viral gene therapies in 10% serum [11]. Viruses, in contrast, generally have efficiencies between 40 and 90% depending on the type of virus and the cell line in question [12].

Ultimately, a trade-off is made. Transfection efficiency can be maximized by using viral vectors, but at a higher risk of complications and higher cost. High doses of naked plasmid can be delivered to offset low transfection efficiency, but if the transgene expression levels are below threshold, the therapy may have no therapeutic value. The relative numbers of non-viral vectors used in preclinical trials are summarized in Figure 1. In a simplistic classification, these vectors are: naked plasmids, lipid-based reagents (which includes liposomes of all varieties, microbubbles, linear lipids which hydrophobically interact with the DNA, and more), polymer-based (which includes dendrimeric systems, polyethyleneimine (PEI), spermine, etc.) and finally cell-mediated, the cells having been transfected prior to implantation.

Figure 1

Distribution of non-viral vectors used in cardiac studies

Do not insert the image in this document; please supply as a separate file.

Naked Plasmids

Naked plasmids, despite having very low transfection efficiencies, have been widely investigated. This is due to a number of factors. Plasmids are relatively inexpensive and simple to prepare, they pose a very low, if not negligible, health risk, and high doses have been observed to have physiological effects *in vivo* [4].

Lipid-based Vectors

Other vectors are, essentially, methods to improve the transfection efficiency of the naked plasmids. Lipid-based vectors – specifically liposomes – are commercially available as transfection reagents (ie. lipofectamine™ and lipofectin™ from Invitrogen, HiFect™ from Lonza, TransPass from New England Biolabs, etc.) [5]. Liposomes exist in a variety of forms, from a single layer of lipids to large multilayered structures. In many ways, liposomes are like vesicles that can be used to encapsulate anything from drugs to plasmids. Liposomes form lipoplexes when they interact with DNA. These lipoplexes can be smaller than 100 nm to larger than 1μm depending on their preparation method and the lipids used. The lipids must have an overall

positive charge to be able to efficiently interact with the negatively charged phospholipids of the cell membrane. This positive charge also promotes interaction with negatively charged nucleic acids. The method of cellular internalization depends partly on the size and partly on the type of lipoplex [13-15].

Polymer-based Vectors

Polymer based vectors are also widely used as transfection reagents. These polymers are cationic – a necessary condition to allow them to condense DNA. The basic interaction between polymers and DNA is electrostatic, as the positive groups on the polymer are attracted to the negatively charged phosphate groups in the nucleic acids. Linear polymers investigated include poly-L-lysine, poly-L-ornithine, polyethyleneimine (PEI), and poly(DL-lactide-co-glycolide) (PLGA) [5, 14, 16]. Dextrans and spermines have also been investigated as carriers and/or condensation agents [17-20]. Branched polymers, including hyperbranched PEI and dendrimers – most commonly polyamidoamine (PAMAM) dendrimers – are being investigated. As DNA is a very large macromolecule, many sections of polymers are generally required to complex a single DNA molecule. Partially degraded dendrimers often give a very effective DNA-polymer complex. This may be because the hyperbranched structure allows high charge concentration in a small area and the degradation process improves the flexibility of the polymer. In general, polymer-nucleic acid complexation can be considered analogous to histone winding of DNA into chromosomes. The efficiency of polymers in condensing DNA depends partly on the polymeric structure and molecular weight, as well as the charge ratio between the negatively charged phosphate groups on the DNA and the positively charged groups in the polymers [16, 21-23].

siRNA

A number of studies using small interfering RNA (siRNA) were cited, pointing to the growing interest in gene silencing for cardiac gene therapy. siRNA is a short sequence of RNA used to bind complementary RNA and mark it for degradation, thereby decreasing the expression levels of the gene coded by that particular sequence [24]. siRNA delivery is analogous to plasmid delivery, as complexation with positively charged polymers or lipids greatly increases the uptake and internalization of the nucleic acids. Complexation with carbon nanotubes or lipid-based systems have been used to improve delivery of siRNAs to the heart [25-27].

Cell-mediated Gene Transfer

Cell-mediated gene transfer is unlike other methods of gene transfer. The cells are transfected ex-vivo and then implanted or injected. This means that the cells expressing the transgene are not necessarily autologous. Furthermore, transfection efficiency is not really an issue because the cells that do take up the gene are selectively cultured and those that do not are eliminated. It is desirable that a vector used for *in vitro* transfection is efficient; it may not be essential.

Sequence Modifications

It is possible to make changes to the sequence of the plasmid itself that may modulate the pattern of transfection. For example, plasmids removed from the capsids of adeno-associated viruses (pAAVs) have been used for transfection of cardiac allografts because the expression period observed with these plasmids is far longer than that of normal plasmids [28]. Promoter sequences can also affect the effectiveness of gene transfer, as ‘powerful’ promoters like cytomegalovirus (pCMV) stimulate high transcription levels of the following gene [29, 30].

Other Considerations

Various physical methods have been used to improve the effectiveness of gene transfer with a number of these vectors. These physical methods include ultrasound, hydrodynamic delivery, and electroporation. Some vectors are more or less effective in transfecting non-dividing cells like cardiomyocytes, which should be a major consideration when selecting the gene delivery method. While a variety of methods have been shown to be capable of transfecting cardiac tissue, it has yet to be made clear what method is the most effective. The lack of standardization in animal models further complicates comparisons.

Comparisons

A number of *in vitro* studies have compared different non-viral vectors. One of the most exhaustive of these was reported by Uchida *et al.* who tested a variety of liposome and polymer formulations, including Lipofectin® and SuperFect™. The effectiveness of the different reagents was shown to be dependent on the cell type, suggesting that different reagents were ideal for different applications. Maximum transfection levels also varied greatly [11]. Considering these findings and the findings of a number of similar studies, there is no clear consensus on the best non-viral vector available for *in vitro* studies. Vector selection for *in vivo* applications is an even more difficult question, and one that remains to be comprehensively studied. However, it has been demonstrated that the use of complexation agents improves the efficacy of gene transfer both *in vitro* and *in vivo* as compared to treatment with naked plasmids.

It is difficult to compare and contrast vector-mediated gene transfer with cell-mediated gene therapy, as there are significant conceptual differences between the two. Vector-mediated gene therapy introduces exogenous genes into native tissue. The cells of that tissue then express the gene and release the resulting protein into the surrounding area. The protein may directly affect the local tissue, or it may be transported to a target area. Cell-mediated gene therapy essentially skips the first step. Cells grown *in vitro* are modified to express the gene in question then implanted into the target tissue where they express the protein. As the cells are already transfected, the transfecting agent will not be injected into the body, and thus is unlikely to cause adverse side-effects. Furthermore, cells expressing the target gene can be grown selectively so that all of the injected cells are transfected. However, the cells are implanted, and thus may be rejected by the body or have reduced viability. The most common technique to address this is to use allogenic cells, which requires more specialization and a more complex treatment procedure. Furthermore, the issue of rejection is not completely eliminated.

Therapeutic Gene

A variety of genes have been investigated for the treatment of heart disease. Some of them address specific pathways that are known to be compromised in damaged hearts while others have multiple effects. In fact, as myocardial tissue is a complex system and the electrochemical properties are closely linked to other metabolic factors, few genes will act exclusively on a single pathway. Genetic engineering of the heart requires detailed knowledge of the roles of many genes, both in healthy and ischemic myocardium. Some of the genes used in clinical and preclinical studies, subdivided by their general function, are discussed below.

Pacemaking

The sinoatrial node of the heart is responsible for generating electrical impulses that control contraction of the cardiac muscle. Irregularities in the generation of these electrical impulses or in their propagation can lead to irregularities in ventricular rhythm. Bradycardia (abnormally slow heart rates), cardiac arrest or damage to heart tissue can result. Electronic pacemakers are the most popular treatment for bradycardia, but biological alternatives, including implantation of cardiac pacemaking cells and genetic engineering of stem cells for implantation, may be far more effective alternatives [31, 32]. One method used to generate pacemaking cells is to induce over-expression of the β-adrenergic receptor [31, 33-36]. Alternatively, delivering a voltage-gated channel protein, specifically the gene for hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels, can induce cells to act as pacemakers [7, 32, 37].

Pacemaking genes can be delivered *in vivo* to induce native cells to act as pacemakers, or cells can be transformed *ex vivo* and then implanted. Furthermore, a variety of cells can be used, including fibroblasts, cardiac cells and stem cells [31, 33-36]. Table 1 provides an overview of preclinical studies investigating gene-therapy based cardiac pacemaking.

Table 1

Summary of reported *in vivo* studies in gene therapy for cardiac pacemaking

Mode of Delivery	Gene	Lead Author	Reference
Cell-mediated	hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels	Potapova	[37]
Naked plasmid	β2-adrenergic receptor	Edelberg	[35]
Naked plasmid	β2-adrenergic receptor	Tomiyasu	[38]
Cell-mediated	β2-adrenergic receptor	Edelberg	[39]

Protection from Ischemia/reperfusion-induced Cell Death

Myocardial damage after MI is due to a combination of the actual ischemic event and the subsequent reperfusion of the tissue. The role of reactive oxygen species in ischemia-reperfusion (IR) injury has been studied for more than 20 years [40]. Oxygen-derived free radicals can cause injury to cardiomyocytes, which leads to contractile dysfunction. Reperfusion is the only viable treatment to prevent irreversible, transmural scarring after infarct, but reperfusion results in oxidative stress. The degree of oxidative stress, and hence myocardial damage, depends on the severity of the ischemic period [41]. Reducing IR damage has been attempted via transfection with genes such as heat shock protein 70 [42-44], heme oxygenase [45, 46] and nuclear factor κB (NFκB) [47]. Antioxidant gene therapy has been attempted with genes like superoxide dismutase (SOD) [48], which were observed to attenuate the damage due to IR injury.

Strongly anti-apoptotic genes, such as bcl-2 [49], and adrenomedullin [50] are other relatively obvious targets for treatment of ischemic tissue, as a large fraction of cells will die via apoptosis in response to ischemia-induced factors. Many of these cells have recovered functionality, and in the case of cardiomyocytes, recovered contractility. Furthermore, implanted cells often suffer significant death rates, due partly to the presence of inflammatory cells and partly to other stresses. Implantation of stem cells transfected with adrenomedullin was observed to both increase the lifetime of the stem cells and significantly improve overall myocardial function [50].

Table 2

Summary of reported *in vivo* gene therapies in reducing cardiac IR damage

Mode of Delivery	Gene	Lead Author	Reference
Lipid-mediated	Heat shock protein (HSP70)	Suzuki	[42]
Lipid-mediated	Heat shock protein (HSP70)	Jayakumar	[43]
Lipid-mediated	Heat shock protein (HSP70)	Jayakumar	[44]
Naked plasmid	Human heme oxygenase-1 (hHO-1)	Tang	[46]
Naked plasmid	Human heme oxygenase-1 (hHO-1)	Tang	[45]
Lipid-mediated	cis element decoy against NFkB	Sawa	[47]
Naked plasmid	SOD (superoxide dismutase)	Palffy	[48]
Cell-mediated	Adrenomedullin (AM)	Jo Ji	[50]
Cell-mediated	Adrenomedullin (AM)	Nagaya	[51]
Naked plasmid	HIF	De Muinck	[52]

Attenuating the Inflammatory Reaction and Reducing Adverse Remodelling

One of the most obvious effects of ischemia is necrosis of the oxygen-starved cardiomyocytes [53]. Factors released by the dying cells stimulate the complement cascade, which, turn, initiates the inflammatory response [54]. Downregulation of the inflammatory response has been found to decrease damage post-MI. For example, gene therapy with an interleukin-1 receptor antagonist (IL-1RA) was found to improve overall survival rates. The same gene has also been used to decrease allograft rejection [55-57]. Similarly, blocking the action of tumour necrosis factor α (TNFα) with a soluble TNFα receptor (sTNFα-R) significantly reduced the infarct size and improved overall cardiac function [58, 59]. Thus, increased levels of inflammation, characterized by higher leukocyte infiltration and higher levels of pro-inflammatory cytokines can be correlated to decreased survival rates.

Leukaemia inhibitory factor (LIF) delivery and treatment with anti-inflammatory cytokines has been observed to decrease levels of inflammation and adverse remodelling. Indeed, for reducing immune and inflammatory reactions, obvious target genes would be anti-inflammatory cytokines, including IL-10, IL-13, and IL-22. IL-10 is the most investigated of the group. IL-10 gene therapy has been used in a variety of applications, including treatment of collagen-induced arthritis [60, 61], lung-transplantation [62, 63], and pancreatic islet transplantation [64], among others. In heart transplantation, a variety of IL-10 plasmids have been employed. Improvements in the allograft survival were observed in all cases, although the magnitude of the improvement is variable [28, 65-67]. Combined gene therapy with IL-4 and IL-10 has been found to be an effective method for protecting cardiac allografts from rejection. A number of other cytokines produced by T-lymphocytes have been studied as well, including IL-13 and IL-22 [68] [69]. Table 3 gives a detailed overview of the *in vivo* gene therapy studies attempting to down-regulate the inflammatory response.

A major component of this adverse remodelling is that the normal cardiac extracellular matrix is replaced with immature collagen type III, which matures to type I collagen [41]. This collagen I matrix is mostly acellular and fibrotic. It is mechanically weaker than the surrounding tissue, and thus vulnerable to systolic stretch. The pumping ability is also reduced, as the function of affected cardiac muscle fibres is compromised [1]. The changes in the extracellular matrix in the remodelled area may also contribute to the loss of contractile function [70]. For example, delivery of a gene encoding human elastin has been found to improve overall cardiac function, reducing scar expansion and thus preventing ventricular enlargement after MI. This seems likely to be a result of changing the composition of the extracellular matrix [71].

In the area of promoting allograft survival, cytotoxic T-lymphocyte antigen 4-immunoglobulin (CTLA4) has also been demonstrated to be an effective gene therapy [72-74]. It is hypothesized that the beneficial effects rely on the decreased infiltration of T-lymphocytes into the allografted tissue [72]. Downregulation of the expression of the transcription factor E2F with a double-stranded decoy gene is another novel strategy that has been employed to improve allograft survival [75]. Finally, transfection with MCH class I antigens was found to decrease hyperacute rejection of transplanted hearts, ultimately improving implant survival [28, 76].

Table 3

Summary of *in vivo* studies on reducing the inflammatory response and adverse remodelling post MI

Mode of Delivery	Gene	Lead Author	Reference
Naked plasmid	TNF-alpha receptor 1 (sTNFR1)	Sugano	[58]
Naked plasmid	IL-1 receptor antagonist	Lim	[55]
Naked plasmid	IL-1 receptor antagonist	Liu	[56]
Naked plasmid	Human IL-1 receptor antagonist	Nakano	[57]

Naked plasmid	Leukaemia inhibitory factor (LIF)	Zou	[77]
Cell-mediated	Elastin	Mizuno	[71]
Cell-mediated	Elastin	Mizuno	[70]
Lipid-mediated	anti-angiotensin converting enzyme (ACE) siRNA	Kim	[25]
Lipid-mediated	IL-4	Furukawa; Furukawa	[78, 79]
Lipid-mediated	IL-10	Sen, Hong, Oshima, Furukawa; Oshima; Furkawa; Oshima	[65-67, 78-80]
Naked plasmid	IL-10	Chang, Doenecke, Palanyandi; Nakano;	[28, 57, 81, 82]
Naked plasmid	IL-13	Elnaggar	[68]
Naked plasmid	IL-22	Chang	[69]
Naked plasmid	Cytotoxic T-lymphocyte antigen 4-immunoglobulin (CTLA4)	Takekubo	[72]
Naked plasmid	Cytotoxic T-lymphocyte antigen 4-immunoglobulin (CTLA4)	Abe	[73]
Naked plasmid	Cytotoxic T-lymphocyte antigen 4-immunoglobulin (CTLA4)	Matsuno	[74]
Lipid-mediated	Antisense cyclin-depended kinase cdk2, E2F decoy	Kawauchi	[75]
Cell-mediated	MHC class I	Geissler	[76]
Naked plasmid	MHC class I	Doenecke	[28]

Angiogenesis

Another important component of the normal inflammatory response is the release of angiogenic factors, which promote angiogenesis in the acute and early healing phases. However, these vessels, if they survive the granulation tissue phase, are insufficient. Thus inadequate angiogenesis can be considered a major component of adverse cardiac tissue remodelling [53].

VEGF Isoforms

There are four forms of VEGF reported in the literature for use in myocardial gene therapy. VEGF-A165 is the most common isoform found in the body, and is highly expressed in the brain, liver, kidneys, cartilage, and muscles [83]. It is also the most commonly used gene in angiogenesis studies. VEGF-A121 is the only other of the six VEGF-A isoforms that has been reported in cardiac gene therapy studies. However, VEGF-A121 has been reported to have 10 to 100 times lower endothelial cell mitogenic activity than VEGF-A165 [84], so comparing studies with genes encoding different isoforms is difficult. VEGF-2 (also know as VEGF-C) [85] and VEGF-D [86] have also been used. VEGF-C has demonstrated relative potency in stimulating angiogenesis in rabbit ischemic hind-limb models, among others, but there is not a significant

body of evidence supporting the use of VEGF-C over VEGF-A165. VEGF-D is expressed in a variety of adult tissues, including the liver, lungs, and, of most relevance, in the heart [84]. In a study on ischemic hind limb muscle, VEGF-D was reported to be the most potent angiogenic factor compared to a variety of other VEGF isoforms [87]. Thus, the use of VEGF-D over VEGF-A165 seems valid, although it still makes it difficult, if not impossible, to compare different treatment methods, doses, or transfection reagents. Without the ability to compare studies, selection of optimal treatment parameters is very difficult, and progress is seriously hampered. Another factor that must be considered is the risk of serious adverse effects associated with VEGF gene therapy, specifically oedema around the heart, as observed in the GENASIS trial [88].

Other Factors and Peptides

To further complicate matters, a variety of other growth factors with potent angiogenic properties have been used. This includes human growth hormone (hGH), hepatic growth factor (HGF), insulin-like growth factor (IGF), platelet-derived endothelial cell growth factor, and stromal cell derived factor (SDF-1). Furthermore, angiopoietin-1 (Ang-1), endothelial nitric oxide synthase (eNOS), PR-39, a proline-arginine rich angiogenic response peptide, and pleiotrophin (PTN) all play roles in IR injury and subsequent revascularization.

hGH is a very potent hormone, which is capable of direct action as well as promoting production of VEGFs and other angiogenic factors. Rong *et al* demonstrated significantly improved ejection fraction, increased angiogenesis, and decreased post-infarct damage after gene therapy with hGH [89]. While the overall function is the ultimate focus of such studies, the ability of hGH to stimulate production of other factors makes it very difficult to separate the effects of the hormone itself with that of downstream effector molecules.

HGF, while not the most obvious choice for use in cardiac gene therapy, has actually been demonstrated to be very effective. Aside from angiogenic action, it has anti-apoptotic effects via bcl-2 overexpression, can protect from oxidizing damage via neutralization of hydroxyl radicals, and may decrease the size of the fibrotic scar [90]. A number of non-viral studies in the heart have demonstrated decreased scar area, increased capillary density and thus myocardial perfusion [91, 92] as well as improved left ventricular function [93], and decreased levels of apoptosis [94]. HGF gene transfer has been demonstrated to be effective in human ischemia, in a clinical study on patients with critical limb ischemia [95]. While no studies in the heart have yet been approved, the success of HGF in pre-clinical myocardial studies recommends it as a very potent alternative to VEGFs. However, a properly matched set of trials with consistent controls and dosages would be required to conclusively recommend one gene over the other.

Without exhaustively examining the remaining factors, it is clear that a pattern emerges. IGF-1[96, 97], PD-ECGF [98], and SDF-1 [99] were all observed to have potent angiogenic potential in addition to other cardioprotective properties, but direct comparison between the studies is difficult.

Ang-1 [100], eNOS [101], PR-39 [52], and PTN [102], sometimes in combination with other pro-angiogenic factors, were also observed to have potent angiogenic effects. However, once again, it is difficult to determine how the improvement in angiogenesis and overall cardiac function in the various studies compare, and how those results compare with studies using VEGFs or other growth factors.

Table 4 contains a detailed overview summarizing the vectors and genes employed to encourage *in vivo* angiogenesis. As a number of clinical trials have been conducted in the area, the trial type is also specified.

Table 4

Summary of *in vivo* cardiac angiogenic gene therapy studies

Mode of Delivery	Gene	Trial type	Lead Author	Reference
Cell-mediated	VEGF-A165 and Ang-1	Preclinical	Ye	[103]
Cell-mediated	VEGF-A165	Preclinical	Suzuki	[104]
Cell-mediated	HGF	Preclinical	Miyagawa	[105]
Cell-mediated or Lipid-mediated	VEGF-A165	Preclinical	Yang	[106]
Lipid-mediated	eNOS	Preclinical	Iwata	[101]
Lipid-mediated	VEGF-A165	Preclinical	Pelisek	[107]
Lipid-mediated	VEGF-A165	Clinical	Hedman	[108]
Lipid-mediated	VEGF-A121	Preclinical	Wang	[109]
Naked plasmid	HGF	Preclinical	Azuma	[91]
Naked plasmid	HGF	Preclinical	Saeed	[110]
Naked plasmid	HGF	Preclinical	Shirakawa	[111]
Naked plasmid	IGF-1	Preclinical	Serose	[96]
Naked plasmid	IGF-1	Preclinical	Liu	[97]
Naked plasmid	Platelet-derived endothelial cell growth factor (PD-ECGF)	Preclinical	Li	[98]
Naked plasmid	Pleiotrophin (PTN)	Preclinical	Christman	[102]
Naked plasmid	SDF-1 (stromal cell-derived factor)	Preclinical	Tang	[99]
Naked plasmid	VEGF-A121	Preclinical	Ojalvo	[112]
Naked plasmid	VEGF-A165	Clinical	Ripa	[113]
Naked plasmid	VEGF-A165	Preclinical	Son	[114]
Naked plasmid	VEGF-A165	Clinical	Gyongyosi	[115]
Naked plasmid	VEGF-A165	Preclinical	Yoon	[116]
Naked plasmid	VEGF-A165	Clinical	Kastrup	[117]
Naked plasmid	VEGF-A165	Preclinical	Radke	[118]
Naked plasmid	VEGF-A165	Preclinical	Sarkar	[119]
Naked plasmid	VEGF-A165	Preclinical	Kloner	[120]
Naked plasmid	VEGF-A165	Preclinical	Schwarz	[121]
Naked plasmid	VEGF-A165	Clinical	Losordo	[122]
Naked plasmid	VEGF-A165	Preclinical	Rutanen	[86]
Naked plasmid	VEGF-A165 and GSF (granulocyte stimulating factor)	Clinical	Wang	[123]
Naked plasmid	VEGF-C	Clinical	Vale	[85]

Administration Technique

The importance of the administration technique should not be underestimated, as the efficiency of transfection will partly depend on the manner in which the solution containing the gene is introduced into the heart. Minimally invasive techniques are generally favoured due to the acceptable safety profile in patients post myocardial infraction. However, there are many trials that work with no-option patients who are undergoing coronary bypass or other procedures requiring thoracotomy, in which case the delivery of the genes is a relatively low impact addition to the overall surgery

Direct Administration

Injection of plasmids or complexes directly into the heart tissue has been used in both pre-clinical and clinical trials [124]. One technique requires direct injections into the ventricular muscle during open heart surgery, which is generally only acceptable if the patient requires the surgery for medical reasons. Delivery via catheters, and possibly thoracoscopic delivery are appealing alternatives, as they are less invasive and would be feasible for use in patients who do not require any sort of surgical intervention.

Thoracotomy

The most straightforward method described in the literature for introducing exogenous genes to the myocardium is direct injection into the cardiac muscle. Many preclinical trials employ this technique, as other methods would be very difficult in small animals. Furthermore, if the MI has been induced by coronary artery ligation, the heart is already exposed. This technique has definite advantages, as a physician can directly observe the beating heart and select areas in need of treatment [112, 122, 125].

Catheters

Catheter based gene delivery, whether via a coronary artery or via a ventricle, can be preformed minimally invasively. The procedure can be guided, like the EUROINJECT-ONE trial, which used the electrochemical mapping NOGA-MyoStar system (Cordis Corp., Miami Lakes, Florida) to deliver plasmids to areas with low perfusion post-MI [115, 117]. Advantages of this technique include that the plasmids can be delivered directly to the damaged areas [124]. Improved therapeutic outcomes might be a result of this improved targeting of the therapy to the compromised areas.

Thoracoscopic

Another possible method of directly introducing the genes to the ventricular muscle would be via thoracoscopic surgery. Briefly, a small incision is made between two rib bones, and a catheter inserted to allow direct access to the heart. However, this technique has not been commonly described in the literature as of yet, and thus its full range of benefits and drawbacks is hard to describe.

Intracoronary Infusion

Intracoronary delivery is the main alternative to intramyocardial delivery. As with catheter based direct injection, it can be considered minimally invasive [124]. However, the delivery is significantly less specific than direct intramyocardial injections, because the DNA is more likely to be carried through the bloodstream to other tissues. Furthermore, the transfection efficiency is quite low, even for viruses, and patients who have coronary artery disease or other complications have even lower transfection efficiencies due to plaque or other barriers to diffusion [124]. The specificity can be very important, because overexpression of certain genes might be very beneficial in heart tissue, but lethal in other organs. A slight modification of this technique employs a balloon that blocks the coronary artery downstream of the vector administration site, increasing the diffusion time and thus the transfection efficiency and specificity [126]. Inducing a

second MI in the course of administering a treatment to improve the first may be acceptable in preclinical studies, but not in humans.

Dose

The dose of gene therapy agent will, in most cases, vary inversely with the transfection efficiency – vectors with high efficiency require very low doses to be effective, while inefficient vectors require significantly higher doses. Comparing the doses between non-viral and viral techniques is difficult as the actual quantity of DNA is not directly related to viral transfection efficiency. For example, 50 μg of naked plasmid DNA [127] was reported as a optimal dose in one preclinical study but in a similar study, $3*10^8$ pfu of adenovirus (which would generally work out to be less than 10 ng of DNA carried by the viruses) was delivered [63]. The virus is thus delivering less than three orders of magnitude the dose of genetic material than the naked plasmids, and yet despite that significant decrease, the transfection efficiency is higher with the adenovirus system. It is likely that an inefficient gene delivery system may be adequate if the therapeutic gene is both potent and secreted from the transfected cell.

Thresholds are a major consideration in drug delivery and design. It is well known that certain drugs, below a threshold dose, have minimal physiological effect. Above that threshold, they are able to perform the task they were administered to perform. Similarly, when gene therapy vectors transfect a certain percentage of cells which will then produce the transgene, the transgene levels must be above a threshold to have therapeutic effect. Threshold values are also important in determining transfection, especially with non-viral vectors. It has been observed that the *in vitro* dose response to non-viral transfection is not linear but has both a low and a high threshold. Specifically, below a certain dose, no transfection is seen. Above that dose, significant transfection is observed. If that dose is increased too much, no transfection is observed. This is especially true with transfection reagents such as polymers [10].

The threshold problem may actually be of significance, as it offers one possible explanation for the observed lack of efficacy in clinical trials that does not translate from the very promising preclinical studies. When considering the dose used, it may not be sufficient to consider only the amount of DNA delivered, because the differences in mass between humans, pigs, and rats, for example, is significant. Table 5 details the average doses used in these three species, then the dose with respect total mass, and the dose with respect to heart mass. There is no significant difference between the doses used in different species, but the dose/total mass used in rats is more than 300x the dose/total mass used in humans. It is possible that the threshold was reached in the rat studies, but not in the human trials, thus explaining the disparity in the therapeutic outcomes.

Table 5

Summary of doses used in selected preclinical and clinical studies

Species	Average Dose [mg]	Dose/total mass [mg/kg]	Dose/heart mass [mg/g]	References
Rat	0.77±0.65	2.486±1.89	0.91±0.66	[101, 102, 109, 114, 116, 119-122]
Pig	0.84±0.66	0.029±0.022	0.0045±0.0036	[86, 91, 107, 110, 112, 118]
Human	0.62±0.63	0.008±0.0079	0.0020±0.0020	[85, 108, 113, 115, 117, 122, 123]

Clinical trials

As of the end of 2008, about 46% of the gene therapy clinical trials involving cardiac tissue used non-viral vectors. The breakdown of that fraction is shown in Figure 2. Naked plasmid is by far the most common non-viral technique, followed by lipid-based vectors and finally cellular vectors. Adenovirus and adeno-associated viruses have also been extensively investigated. No polymers have been used in clinical trials in the heart as of yet, although pre-clinical evidence suggests they may be very effective.

Figure 2

Distribution of vectors used in cardiac gene therapy clinical trials

Do not insert the image in this document; please supply as a separate file.

Of the non-viral clinical trials, all of the genes investigated have had some relation to angiogenesis. More than 90% of these trials have used a VEGF isoform. HGF, FGF, and NOS have also been investigated. The EUROINJECT trials, one of the biggest non-viral gene delivery clinical trials, delivered VEGF-A165 intramyocardially [115, 117, 128]. Perfusion appeared to be enhanced, anginal class improved, and exercise capacity extended [129]. However, stress-induced myocardial perfusion abnormalities did not appear to be improved over placebo [117]. Phase I clinical trials with HGF in patients with critical limb ischemia have thus far demonstrated safety [95], although further trials will be required to show therapeutic benefits. Furthermore, success in extremities does not necessarily correspond to success in the myocardium, which is a significantly more complex system.

Viral clinical trials have examined a host of other genes, including a variety of fibroblast growth factors (FGF-2 and FGF-4), and sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) [130]. While little attention has been given to these genes in non-viral trials, either clinical or pre-clinical, they have significant therapeutic potential. Furthermore, most of the genes investigated in preclinical trials remain largely untested in humans despite promising results in a variety of animal models.

While few clinical trials have established major benefits as a result of cardiac gene therapy, the safety of plasmid gene delivery has been comprehensively demonstrated. Several phase I and II trials have been conducted (for example, the EUROINJECT-ONE trial). No acute complications were reported, and a one-year follow-up of a Phase I trial of direct delivery of pVEGF-C to the heart found no complications as a result of the treatment [125]. In fact, no ill-effects were reported as a result of non-viral gene therapy in any of the trials cited, either with naked or lipid-complexed plasmids [85, 108, 113, 115, 117, 122, 123, 129]. As a result, most papers detailing phase I/II trials recommend further trials, many of which are now on-going [129].

Future directions

While much progress has, indeed, been made over the last few decades, non-viral cardiac gene therapy is still in its infancy. No consensus has been reached with regards to the optimal genes for given applications, nor even the optimal isoforms of those genes. As a case in point, there are four isoforms of VEGF being investigated. Nor is VEGF necessarily the most appropriate angiogenic growth factor – evidence exists to suggest that HGF or FGF-4 may, in fact, be better for revascularization in cardiac settings as the effects of VEGF are more related to capillary formation while HGF and FGF-4 encourage growth of arterioles, which may be more effective for revascularization of ischemic tissue. Part of the motivation for using different isoforms is not at all scientific, but practical – due to access to a particular plasmid or intellectual property considerations. Cell-mediated therapy is even less standardized, as a third variable – cell type – is introduced into the equation. Direct comparisons, in the same models and with optimized dosages, could possibly provide the information required to give cardiac gene therapy clinical relevance.

The transfection efficiency of plasmid DNA remains another major hindrance to the progress of non-viral gene therapy. However, in pre-clinical studies, the success of transfection reagents suggests this problem may be at least partly addressed [78]. Furthermore, only a few transfection reagents have actually been tested in the heart, leaving even more room for future studies. While most recent work uses therapeutic genes, a comprehensive comparison of different non-viral vectors delivering a reporter gene to the myocardium could be extremely valuable. It has been demonstrated that the efficacy of different vectors varies greatly between cell types *in vitro* [11], an effect likely to be even more significant *in vivo*.

Comprehensive dose response studies may demonstrate the threshold effect, and thus explain the relative success and failure of preclinical vs. clinical studies respectively. As has been shown in Table 5, the plasmid doses used in humans are not significantly higher than the doses used in rats. Relevance of this dosing regimen is questionable as rats have a much smaller heart size and total body mass. Again, the use of reporter genes may be valuable in this instance as the goal is comprehensive quantification of transfection and protein expression.

It may be that a multidisciplinary approach to cardiac gene therapy may provide another avenue for future improvements. A stronger connection between engineering and medicine, for example, could introduce gene delivery systems better optimized for therapeutically appropriate delivery techniques.

Conclusion

More than ten years after Isner et al. initiated the first clinical trial using a VEGF plasmid to treat restenosis; myocardial clinical studies have failed to demonstrate major benefits of non-viral gene delivery. The safety of naked plasmid use has been well described, and modest benefits have been observed, but not on the scale that was expected. This failure is not only due to insufficient transfection efficiency, as very limited success has been observed in viral trials, which should have far higher efficiencies. Improved delivery, whether via complexed DNA or implanted genetically engineered cells, might result in clinically relevant progress, but the likely benefits of proper optimization of all aspects of the therapies cannot be overlooked.

Specifically, appropriate attention to all three facets of cardiac gene therapy – vector, therapeutic gene and administration technique – must be provided. Using a less efficient administration technique with non-viral vectors might result in negligible transfection levels and thus no therapeutic effect even though the gene and vector would have been a potent combination with another administration technique. Similarly, using an effective vector and administration technique to deliver a gene with very little therapeutic value is unlikely to improve overall cardiac function. Adopting an engineering approach to gene therapy in the myocardium may prove to be the most effective way to develop clinically relevant treatments.

To genetically engineer the recovery of damaged myocardium, the optimal vectors, genes, doses and administration techniques must be combined. Proper optimization will require that standards be selected, and used across the board. With some luck, it may be that an engineering approach will at last allow full realization of the potential of myocardial gene therapy.

Notes

The authors would like to acknowledge Science Foundation Ireland, Research Frontiers Program under Grant Number (06/RFP/ENM005) for funding of this project.

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