Introduction

In vivo gene therapy is a strategy in which nucleic acid, usually in the form of DNA, is administered to modify the genetic repertoire of target cells for therapeutic purposes. This technology is now being evaluated in clinical trials as a treatment for genetic disorders, and is also being considered as a potential treatment of acquired diseases, including atherosclerotic arterial disease, restenosis after vascular interventions, and cardiac allograft rejection. In this review we will focus on the most recent developments of gene transfer technologies into the cardiovascular system, and then on the main potential gene therapy applications for cardiovascular disease.

How can gene transfer into the cardiovascular system be achieved?

Table 1

Gene therapy implies the effective transfer of genes to the human body in a manner that is safe or at least has acceptable associated risks, does not invoke immune responses or pathological processes, and expresses the therapeutic gene appropriately to achieve a therapeutic outcome. Cells are capable of taking up genetic information on their own, but as this process is very inefficient, various vectors have been developed to enhance this process. These gene transfer vectors can be classified as non-viral and viral. In non-viral vectors, DNA is complexed or conjugated to molecules that facilitate DNA penetration into the cell. The major advantage of non-viral vectors is to avoid the use of infectious agents. However, their use is limited since they are not very efficient in vivo. One non-viral strategy is to complex DNA with liposomes (i.e. positively or negatively charged lipid molecules). Although not completely understood, liposome-mediated enhanced DNA uptake by cells seems to occur via non-specific fusion of the DNA-liposome complex with cell membranes. Another non-viral strategy is to conjugate DNA to a protein that functions as a ligand for a cell surface receptor. The ligand–receptor interactions allow the DNA to enter the cell as the ligand is internalized. One potential advantage of this approach is the ability to target specific cell types based upon their receptors. The complex physical structure of this type of vector may lead to an immunological response to the complex or its individual components, which represents a potential adverse effect.

Viral vectors capitalize on the natural ability of viruses to enter and subsequently to modify the genetic programme of the host cells. In viral vectors, the new gene is incorporated into the viral genome and the recombinant virus is engineered to infect cells, express the new gene, but does not reproduce itself within the host. Much of the work with viral vectors has focused upon retroviruses, (i.e. RNA viruses) which enter cells through specific surface receptors. Once inside the cell, retroviral RNA is converted to DNA which then randomly integrates into the host cell genome. Since integration occurs in proliferate cells, recombinant retroviruses have been primarily used for ex vivo strategies: cells are removed from the recipient, and are stimulated to proliferate ex vivo, and then the modified cells are returned to the recipient. This requirement for host cell proliferation is an obstacle to efficient in vivo gene transfer in the cardiovascular system because of the slow cell turnover rate in situ, such as in endothelial cells or in cardiomyocytes.

In contrast to retroviral vectors, recombinant adenoviral vectors provide a promising approach for in vivo gene therapy applied to cardiovascular disease. These vectors are based upon adenoviruses (i.e. 36 kb double stranded DNA viruses) which have a broad host range. Adenoviruses (Ad) enter cells through specific surface receptors and travel to the nucleus where, in contrast to retroviruses, the DNA rarely integrates into the genome. Importantly for gene therapy, adenoviruses do not require host cell proliferation for successful gene transfer and expression.

For gene therapy applications, adenoviruses are rendered replication deficient by removing a portion of
Table 1  Gene transfer vectors

<table>
<thead>
<tr>
<th>Vector</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Plasmids-liposomes</td>
<td>Non immunogenic</td>
<td>Low efficiency</td>
</tr>
<tr>
<td></td>
<td>Easy to produce</td>
<td>No integration into host cell genome</td>
</tr>
<tr>
<td></td>
<td>No DNA size limit</td>
<td></td>
</tr>
<tr>
<td>DNA-proteins complexes</td>
<td>No live virus</td>
<td>In vivo efficiency unknown</td>
</tr>
<tr>
<td></td>
<td>No DNA size limit</td>
<td>Duration of expression unknown</td>
</tr>
<tr>
<td>Retrovirus</td>
<td>Well known</td>
<td>Works only in dividing cells</td>
</tr>
<tr>
<td></td>
<td>Long duration of expression</td>
<td>Foreign gene must be &lt; 8 Kb</td>
</tr>
<tr>
<td></td>
<td>Integration into host cell genome</td>
<td>Low viral titre (10^10/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Associated with lymphoma in monkeys</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Works in differentiated cells</td>
<td>No integration into host cell genome</td>
</tr>
<tr>
<td></td>
<td>High in vivo efficiency</td>
<td>Complicated viral structure</td>
</tr>
<tr>
<td></td>
<td>High viral titre (10^9/ml)</td>
<td>Potentially pathogenic</td>
</tr>
<tr>
<td>Adeno-associated virus</td>
<td>High in vivo efficiency</td>
<td>Strong immune reaction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Short duration of expression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Foreign gene must be &lt; 8 Kb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Might recombine with wild adenovirus</td>
</tr>
<tr>
<td>Herpes virus</td>
<td>High in vivo efficiency</td>
<td>Not used yet in cardiovascular system</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Potential contamination with adenovirus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Integration into host cell genome unclear</td>
</tr>
</tbody>
</table>

EI, the region of the viral genome responsible for regulation of adenoviral replication[17]. The adenoviral genome is then engineered to accommodate a new gene with appropriate controlling elements. The resulting recombinant adenoviral vector can infect target cells, express the new gene, but it will not replicate because of the absence of the EI function. Recombinant adenoviral vectors have been successfully used in vivo to transfer genes into several organs and cell types, including respiratory airway epithelial cells, hepatocytes, endothelial cells, skeletal muscle cells, cardiac myocytes, mesothelial cells, salivary gland cells, synoviocytes, and neurons (for review, see[81]). Potential disadvantages of recombinant adenoviral (Ad) vectors include the possibility that low level viral replication and viral gene expression may occur[49]. Current replication-deficient Ad vectors have been reported to express viral proteins that could induce cellular immune responses leading to the destruction of the infected cells and subsequent repopulation of the organ with non-transduced cells[20]. Exposure of normal arteries to adenovirus vectors in rabbits results in infiltration of T cells throughout the artery wall, upregulation of adhesion molecules, and small neointimal hyperplasia[21]. Work is being done to stop the EI-deleted virus from expressing viral proteins, and to increase the amount of exogenous DNA that can be transduced using Ad vectors[15,22]. Another potential disadvantage of adenovirus vectors is their necessary repetitive administration because of the lack of integration of the exogenous gene into the host cell genome; if there is an immunological response of the host to the vector, repetitive administration may be prevented by the immune system. Studies have been carried out to evaluate whether repeated inoculation with adenovirus vectors results in an inflammatory immune response and reestablishment of therapeutic protein levels, and have yielded conflicting results[23,24]. Finally, further investigations are required to evaluate the efficacy and safety of adenovirus vectors. Results to date suggest that EI-deleted adenoviruses are unable to replicate in vivo and only mediate toxic effects when administered in high doses. This supports the continued development of adenovirus vectors for use in gene therapy[11].

**Antisense oligonucleotides**

A great deal of interest has been focused on the potential for developing human therapeutics based on antisense oligonucleotide strategies. Oligonucleotides that are complementary, or antisense, to individual mRNA sequences bind to the sequence and prevent its translation. Inhibition of several cellular proto-oncogenes, including c-myb, c-myc, non-muscle myosin, cdc-2 kinase, and proliferating cell nuclear antigen (PCNA) have been shown to inhibit smooth muscle cell proliferation in vitro and to inhibit neointimal thickening in vivo in a balloon-injured rat carotid artery model of restenosis[3,25,26] or in vein grafts[28]. The clinical applicability of antisense technology remains limited by a relative lack of specificity, slow uptake across the cell.
membrane, and rapid intracellular degradation of the oligonucleotides[2,3].

What methods have been developed for gene delivery?

The transduction of appropriate target cells represents the critical first step in gene therapy; consequently, the development of gene transfer methods suitable for various forms of therapy has been a major focus of research.

Although the feasibility of in vivo arterial gene transfer is currently accepted, it has also become apparent that with the possible exception of adenovirus vectors, the commonly used methods for in vivo arterial gene transfer are extremely inefficient[28,29]. In addition, use of these vectors may not result in gene transfer into all layers of the arterial wall. In previous work with retroviral vectors, approximately 1 in $10^6$ to 1 in $10^5$ cells were transduced after injection of high-titre retroviral vectors into rabbit aorta by means of a local delivery balloon catheter. Transduced cells were not specifically localized[30]. The transfection efficiency that can be achieved using naked DNA or liposomes is typically less than 1%[30,31]. In these studies, transduced cells were limited to the neointima. Other groups have reported detection of arterial gene transfer only by using the extremely sensitive luciferase reporter gene, which is capable of detecting expression in fewer than 1 cell out of 10,000[32]. No specific localization of transduced cells has been provided in these studies. Adenovirus vectors have been successfully used in vivo to transfer exogenous genes into a high number of endothelial cells in various animal arteries and veins[33–36]. However, anatomical barriers may limit the distribution of in vivo gene transfer into the arterial wall of normal vessels[37]. In balloon-injured arteries the efficiency of adenovirus vectors is more variable, and may be less in atherosclerotic arteries than in normal arteries[28]. Furthermore, dose-related toxicity has recently been described with adenovirus vectors in a rat model of balloon-injured carotid artery[38].

Similarly, because catheter-based delivery is obviously attractive for eventual clinical application of vascular gene transfer, several methods of local vascular delivery have been evaluated, including direct surgical instillation (dwell), and infusion through perforated or double-balloon catheters[39]. The catheter systems facilitate gene transfer, either by filling the lumen of an isolated vessel segment with the vector and allowing passive contact between the two balloons, or by pressurized diffusion/infusion of the vector into the vessel wall[40]. The strategies used to facilitate vascular gene transfer produce qualitatively different patterns of foreign gene expression. Vessels injected by the dwell or double-balloon techniques show selective genetic modification of vascular endothelium, whereas introduction of virus via high-pressure balloon systems resulted in gene expression primarily in medial smooth muscle cells[36]. In view of the impermeability of the endothelial layer to particles the size of adenovirus (70 to 80 nm), mechanical disruption of the intima may be necessary for gene transfer targeting medial smooth muscle cells[37].

In summary, the efficiency and selectivity of gene transfer into the vessel wall depends on the delivery system used. Specifically, while available approaches appear sufficient for targeting endothelial cells in normal vessels[33,30], the delivery strategies evaluated for targeting cells residing in the media result in less efficient genetic modification[28,37]. Furthermore, since the clinical application of localized delivery of vascular gene therapy concerns sites of balloon angioplasty, the importance of vessel trauma related to local delivery techniques remains controversial. In addition, the degree of tissue injury resulting from this method of delivery also may play a role in impairing foreign gene expression[39].

Much interest has also focused on developing techniques to introduce recombinant genes directly into the heart. Although several studies have demonstrated successful gene transfer into the ventricular myocardium of rats using direct injection of plasmid DNA, the number of myocytes transfected with this method appears to be too small for successful gene therapy application[41,42]. Adenovirus vectors represent an alternative means for introducing genes into the heart. Stratford-Perricaudet et al. first demonstrated direct gene transfer into intact myocardium by adenovirus vectors following the intravenous injection of new-born mice[43]. Direct injection of adenovirus vectors into the ventricular myocardium has been demonstrated to be efficient and safe in both adult rats and adult swine[44,45]. Histochemical analysis showed that the percentage of cardiomyocytes expressing the exogenous gene could be quite high in microscopic regions adjacent to the needle track; however, positive cells were rarely observed farther than 5 mm from the injection site[45]. Furthermore, the adenovirus vector induced pronounced leukocytic infiltration that was far in excess of that seen after injection of vehicle alone. Catheter-mediated infusion of adenovirus vectors into the coronary arterial circulation in vivo in rabbits induces expression of the exogenous gene in both the coronary arteries and the myocardium[46], and appears to be safe in large animals such as pigs[47]. However, the cell-mediated immune response to the adenovirus vector and the limited distribution of exogenous gene expression suggest that less immunogenic recombinant vectors and more homogeneous administration methods will be required before adenovirus vectors can be used to treat cardiovascular diseases[2,15].

What are the potential clinical applications of gene transfer in the cardiovascular field? (Table 2)

The first clinical trial to treat a cardiovascular disorder was initiated at the University of Michigan (U.S.A.).
been focused on inhibiting smooth muscle cell proliferation in the vascular wall. The initial approach has involved small segments of arterial vessels is now available offering a theoretical opportunity to locally modify gene expression in the vascular wall. The initial approach has been focused on inhibiting smooth muscle cell proliferation and associated luminal narrowing were successfully inhibited. Adenovirus-mediated transfer of the retinoblastoma (Rb) gene, whose protein product inhibits cell cycle progression, has also been successfully used in a similar animal model. Von Der Leyen et al. recently used a gene transfer system to link the capsid protein of the Haemagglutinating virus of Japan and liposomes, to express nitric oxide synthase into the vessel wall of balloonized rat carotid arteries. This increased vascular reactivity of the injured vessel and inhibited neointima formation by 70%.[54] Finally, transfer of the gene coding for the angiotensin II type 2 receptor, and of the gene coding for the thrombin inhibitor hirudin also resulted in a reduction in neointima formation in injured rat carotid arteries.[55,56] These strategies may be overshadowed by the recent approach to understanding restenosis: it appears that constrictive remodelling, rather than smooth muscle proliferation, may be the principal mechanism of restenosis after angioplasty.[57] However, the cellular mechanisms underlying remodelling are not clearly understood; therefore the targeted gene(s) to alter remodelling remain to be defined.[58] Gene transfer techniques are also being used to better define the in vivo role of growth factors in restenosis, such as PDGF, TGF, or FGF.[59-61]

In addition to attempts at treating cardiovascular diseases through the secretion of inhibitory factors, gene therapy also offers the potential for treating established acquired diseases by the local expression of stimulatory proteins. In a recent study, Isner et al. reported the induction of functionally significant collateral vessels, transferring the gene encoding vascular endothelial growth factor (VEGF) in a model of ischaemic rabbit hind limb using ligation of the external iliac and femoral arteries; a clinical protocol with injection of naked DNA coding for VEGF has recently been started in the U.S.A.[62], while experimental studies with an adenovirus vector encoding the VEGF gene are in process[63]. This approach holds great promise for the treatment of severe peripheral vascular disease and end-stage ischaemic cardiomyopathies. Somatic gene therapy for complex disorders such as hypertension is also being studied, with encouraging experimental results involving transfer of the gene encoding kalikrein.[64,65] Finally, gene transfer technologies are also being evaluated to prolong cardiac allograft survival[66] or to prevent thrombosis[67,68].

Table 2 Gene therapy for cardiovascular disease

<table>
<thead>
<tr>
<th>Cardiovascular disease</th>
<th>Example of target gene</th>
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<tbody>
<tr>
<td>Familial hypercholesterolaemia</td>
<td>LDL receptor</td>
</tr>
<tr>
<td>Critical limb ischaemia</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>Restenosis after angioplasty</td>
<td>Suicide gene</td>
</tr>
<tr>
<td></td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td></td>
<td>Angiotensin II type 2 receptor</td>
</tr>
<tr>
<td></td>
<td>Hirudin</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>Antioxidant genes</td>
</tr>
<tr>
<td></td>
<td>Heat shock proteins</td>
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<tr>
<td>Transplant rejection</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Kallikrein</td>
</tr>
<tr>
<td>Thrombosis</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td></td>
<td>Cyclooxygenase</td>
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</table>

and involved patients with homozygous familial hypercholesterolaemia, a condition associated with an absence or a deficiency of the hepatic low density lipoprotein (LDL) receptor, and characterized by severe hypercholesterolaemia and premature atherosclerosis.[68] Patients enrolled in the study were treated by ex vivo retrovirus-mediated transfer of the gene encoding the normal human LDL receptor to cultured hepatocytes isolated from a surgically resected segment of the patient's liver. The transfected cells were then reintroduced into the liver by infusion into the portal vein through a catheter left in situ at the time of hepatic resection. In the first treated patient, the procedure was associated with a rapid and sustained reduction in serum LDL, an increase in serum HDL, and a further improvement in lipid profile after treatment with lovastatin. Clearly, if this approach is shown to be feasible and effective in larger studies it may be of clinical relevance in the treatment of patients with the heterozygous form of the disease in whom a moderate degree of hypercholesterolaemia is also associated with premature atherosclerotic coronary and peripheral vascular disease. Other experimental approaches using adenovirus vectors and/or transfer of the ApoE are currently ongoing.[49-51].

The second potential role for gene therapy in the field of cardiology is to inhibit the development or progression of acquired diseases, such as atherosclerosis, arterial thrombosis, or restenosis after angioplasty. Because acquired diseases are multifactorial, this represents a far greater challenge than replacing or increasing deficient proteins associated with inherited single gene defects.

Restenosis is an important target for gene therapy, as it is a frequent and costly complication of balloon angioplasty and/or related techniques, and it has proved refractory to all pharmacological therapies.[59]. Furthermore, the technology to enable gene transfer into small segments of arterial vessels is now available offering a theoretical opportunity to locally modify gene expression in the vascular wall. The initial approach has been focused on inhibiting smooth muscle cell proliferation. Ohno et al. used adenoviral vectors expressing herpes virus thymidine kinase at the site of balloon denudation in the iliac arteries of pigs exposed to the nucleus as analogue ganciclovir; both smooth muscle cell proliferation and associated luminal narrowing were successfully inhibited.[52] Adenovirus-mediated transfer of the retinoblastoma (Rb) gene, whose protein product inhibits cell cycle progression, has also been successfully used in a similar animal model.[53] Von Der Leyen et al. recently used a gene transfer system to link the capsid protein of the Haemagglutinating virus of Japan and liposomes, to express nitric oxide synthase into the vessel wall of balloonized rat carotid arteries. This increased vascular reactivity of the injured vessel and inhibited neointima formation by 70%.[54] Finally, transfer of the gene coding for the angiotensin II type 2 receptor, and of the gene coding for the thrombin inhibitor hirudin also resulted in a reduction in neointima formation in injured rat carotid arteries.[55,56]. These strategies may be overshadowed by the recent approach to understanding restenosis: it appears that constrictive remodelling, rather than smooth muscle proliferation, may be the principal mechanism of restenosis after angioplasty.[57]. However, the cellular mechanisms underlying remodelling are not clearly understood; therefore the targeted gene(s) to alter remodelling remain to be defined.[58]. Gene transfer techniques are also being used to better define the in vivo role of growth factors in restenosis, such as PDGF, TGF, or FGF.[59-61].

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Conclusion

Current data suggest that it might be possible to treat several human diseases by transferring genetic material into specific cells, rather than by conventional drugs. Although the concept may appear straightforward and to be the most direct application of recombinant DNA

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technology, research has indicated that successful implementation of gene transfer in the clinic will require the coordinated development of a variety of new technologies, combined with interactions between investigators from divergent medical and basic science disciplines. As indicated in the recent report on gene therapy ordered by the NIH, clinical efficacy has not yet been definitely demonstrated in any gene therapy protocol, and significant problems remain in all its basic aspects\(^6\). In the field of cardiovascular diseases, besides the technological challenge to improve short-term clues, such as transfection into atherosclerotic arteries, or long-term efficiency, there is also a need to define clear cellular and gene targets.

References


