Defining Gene Transfer Before Expecting Gene Therapy
Putting the Horse Before the Cart

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By all means keep your enthusiasm, but let verification be its constant companion.
Louis Pasteur, 1822 to 1895.

The remarkable advances in our understanding of the molecular and biochemical bases for disease during the past decade, in conjunction with the “genomic revolution,” have generated understandable enthusiasm for the development of genetic therapies. Whereas the initial hope was that gene therapy would aid in the treatment of patients with primary genetic disorders, applications have now been expanded to populations with diseases in which acquired environmental and other factors play a major pathogenic role. Research into gene transfer techniques as potential therapeutic strategies for cardiovascular disease began in the early 1980s and has been translated into phase I and, more recently, phase II and III clinical trials. Currently, 46 of 509 ongoing clinical gene transfer trials are investigating cardiovascular diseases.1 The majority of gene therapy trials and studies in cardiovascular disease are aimed at testing the safety and efficacy of therapeutic angiogenesis, and to a lesser extent, examining restenosis after vascular intervention.

At this juncture, at a time of justifiable excitement and intense interest in the role of gene therapy in cardiovascular disease, it is perhaps opportune to critically review what we have learned and to discuss what directions should be taken in the near future. Gene transfer is, after all, a system of drug delivery that uses complex and potentially toxic biochemicals. Nonetheless, the emphasis of current clinical trials has been on developing individual gene transfer “products” as therapeutic agents, as opposed to focusing on the core components of gene therapy—namely, gene transfer and transgene expression. Although these clinical studies are generating important data on the feasibility and safety of individual gene transfer products, there needs to be a redirection toward the growing need to enhance our understanding of the underpinnings of human gene transfer. Even though defining vector distribution and transgene expression and function have been essential parts of preclinical studies, these same parameters have not been adequately addressed in current clinical studies. Thus, the lack of human pharmaco-

kinetic and pharmacodynamic data will eventually limit the effective application of gene transfer in the treatment of cardiovascular disease. Opportunities to obtain the necessary data will require cooperation, perseverance, and imagination within the cardiovascular gene transfer community. Nonetheless, the acquisition of such data is the foundation on which future clinical studies could flourish or flounder.

Current State of Cardiovascular Gene Transfer

The progress from pioneering studies, which defined the ability to express transgenes in the hearts and vasculature of normal animals, to the development of a phase III clinical study for angiogenesis in patients with stable angina, required more than a decade of research and development. The field progressed by establishing transgene expression, function, and effects in preclinical studies before initiating clinical trials (Figure 1). These defined end points are similarly important in clinical trials.

Studies demonstrating expression and function of virally and nonvirally delivered transgenes to the vasculature soon led to the development of genetic strategies to inhibit neointimal formation after vascular injury.2 Viral gene transfer to the vasculature in humans has been demonstrated in one small study by Laitinen and colleagues3 in Finland. The translation of the results of therapeutic preclinical gene transfer studies into therapies to inhibit neointimal formation in humans was initially limited by a combination of factors, including a lack of effective delivery catheters, safety concerns with adenoviral vectors, and the development of increasingly effective nongenetic therapies. Recent progress in the use of gene transfer to induce angiogenesis in ischemic tissue avoided these major concerns that were associated with the original antirestenosis strategies. Led by the pioneering work of the late Dr Jeffrey Isner, it was hypothesized that potent vascular growth factors might be delivered by plasmid DNA with direct intramuscular injection. This concept, well defined in preclinical studies, has led to a series of clinical trials that have used angiogenic transgenes, including isoforms of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and hypoxia inducible factor-1α.
Originally, these angiogenic studies used plasmid DNA in the setting of peripheral vascular disease and ischemia. Subsequently, there has been an evolution to the use of adenoviral vectors and delivery to the myocardium. Additionally, the clinical scenarios in which these vectors have been tested evolved from patients with end-stage disease to patients with chronic stable angina. Currently, one of the 4 phase III trials of gene transfer underway in the United States involves the intracoronary delivery of a recombinant adenoviral vector expressing FGF-4 in patients with chronic stable angina.

The development of gene transfer procedures has coincided with substantial improvements in standard surgical and medical therapies for cardiovascular disease. The widespread use of coronary stents and brachytherapy, now accompanied by the potential of coated stents, has tempered the enthusiasm for gene therapy for restenosis. Clinical trials with angiogenic peptides may demonstrate efficacy or safety advantages over gene transfer strategies for angiogenesis. Thus, in the setting of this rapid development of genetic and standard therapeutics, it is particularly relevant to emphasize that the basic principles of pharmacokinetics and pharmacodynamics should not be ignored.

Pharmacokinetics of Gene Transfer
Pharmacokinetics is the study of biodistribution, excretion, and biotransformation of drugs. The 1995 National Institutes of Health report on gene therapy concluded that “interpretation of the results of many gene therapy protocols has been hindered by a very low frequency of gene transfer, and reliance on qualitative rather than quantitative assessments of gene transfer and expression.” Six years later, there is a growing but nonetheless insufficient body of quantitative information about the pharmacokinetics of gene transfer vectors and their expressed transgenes in humans. Areas in which clinical findings are needed include the biodistribution of vectors and the kinetics of transgene expression (Figure 2). Although these data may be difficult to obtain because of inherent technical limitations and ethical considerations in obtaining tissue in patients, such knowledge is essential to ensure the safe use of gene transfer vectors and their translation to effective clinical practice.

Biodistribution and Excretion
In cardiovascular gene transfer, the delivery of a vector to target tissues generally has involved direct physical methods, including local perfusion of vascular territories and direct injection. The potential exists, however, for the wider distribution of the vector, resulting in untoward effects on distant organs. Biodistribution data from clinical adenoviral delivery studies to the heart are beginning to emerge. After intracoronary delivery of a recombinant adenoviral vector encoding FGF-4, up to 13% of the delivered dose (3.2×10^10 viral particles delivered) was found in the pulmonary arterial circulation, and at the highest dose, adenovirus was detected in the systemic venous circulation. Notably, preclinical studies with a vector expressing FGF-5 had previously shown only 1.7% of a dose (2×10^11 viral particles delivered) in the pulmonary circulation, and a very short half-life (2 minutes) of adenoviral vectors in the circulation. These findings highlight the importance of further defining biodistribution in humans.

Data from ongoing clinical studies are beginning to define the pattern of excretion of adenoviral vectors. In the FGF-4 clinical study that used intracoronary delivery, no adenovirus was seen in patient semen obtained 8 weeks after delivery. Direct intramyocardial injection of adenovirus expressing VEGF was not associated with systemic effects, and viral shedding was not observed in nose, throat, urine, or blood samples. Whether these original findings can be extrapolated to other clinical scenarios or vectors, including vascular delivery to peripheral beds, is uncertain. In fact, extrapolation of adenoviral biodistribution data to other vectors is not possible, as demonstrated by the fact that intra-arterial delivery of adenovirus-associated virus has been associated with the subsequent presence of viral DNA in semen, which is contrary to data from studies with adenoviral vectors.

There is a paucity of data from clinical studies evaluating the biodistribution of plasmids. Accurately defining the

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**Figure 1.** The development of cardiovascular clinical gene therapy. Preclinical studies define transgene expression and function before testing in animal models of disease. To develop effective clinical gene therapy, similar data defining the expression and function of transgenes will be required.

**Figure 2.** Pharmacokinetics of gene transfer. Biotransformation is required for transgene product expression and function. The biodistribution and excretion of the vector and product contribute to the safety and efficacy of the gene transfer strategy.
biodistribution and excretion of gene transfer vectors, even plasmid DNA, is essential for the safe progression of clinical gene transfer studies. Clinical studies with plasmid DNA delivered intratumorally\(^1\) failed to demonstrate plasmid in serum after delivery. The inability to routinely obtain tissue for biodistribution studies in humans might be compensated for by routinely obtaining all available bodily fluids, including semen, in all subjects, with rigorous analysis of biopsy or autopsy tissue when available.

Another factor that might affect the biodistribution of transgene products is the inherent biodistribution of the proteins themselves, including interactions with other local and circulating factors or with concomitantly administered drugs. For instance, there is a complex relationship between VEGF, VEGF receptors, and heparansulfate or heparin. Tofukuji and coworkers\(^1\) have shown in a porcine model of VEGF, VEGF receptors, and heparansulfate or heparin. and circulating factors or with concomitantly administered transgene products is the inherent biodistribution of the autopsy tissue when available.

For VEGF on transgene product function are not available. To furthermore, data about the effect of expression of the receptors for VEGF on transgene product function are not available. To what extent these interactions are clinically relevant to the biodistribution of genes encoding growth factors remains to be established.

**Biotransformation**

Biotransformation is the net effect of the biochemical reactions on a pharmaceutical compound. These may result in inactivation or, less commonly, activation of the compound. The effective biotransformation of the gene sequences packaged in any vector into a potentially therapeutic transgene product is an essential component of gene transfer strategies. This process utilizes the innate transcriptional and translational machinery present within target cells, and its kinetics depends on vector dose, regulatory elements, and host response. For gene therapy to be successful, it is essential that the time course of transgene expression be appropriate for the disorder to be treated. Brief periods of transgene expression (hours or days) may be sufficient to inhibit activation and adhesion of monocytes and platelets and to regulate cellular proliferation after acute vascular injury. Chronic expression may be required for the long-term treatment of myocardial dysfunction.

Novel mechanisms to target transgene expression by limiting its expression to specific cell types include transcriptional targeting. This strategy uses cell-specific regulatory elements to drive transgene expression. In the vasculature, targeting has been accomplished by the use of smooth muscle cell–specific promoters such as SM22α to drive transgene expression.\(^1\) This strategy has the theoretical advantage of avoiding transgene expression in antigen presenting cells that might otherwise hasten immune surveillance and limitation of transgene expression.

**Angiogenesis Studies**

The majority of transgene expression data comes from angiogenesis trials. Measurements of circulating VEGF levels after gene transfer have yielded conflicting results. In one report, there was a transient and marginal increase in serum VEGF\(_{165}\) with a peak at 1 to 3 weeks after intramuscular injection of plasmid encoding for VEGF.\(^1\) A later report from the same group showed an up to 3-fold increase in plasma levels of VEGF\(_{165}\) that persisted for at least 28 days.\(^1\) A similar 2- to 3-fold increase in plasma VEGF\(_{165}\) levels was reported in 13 patients undergoing direct intramyocardial gene therapy, using the same plasmid, for chronic myocardial ischemia.\(^1\) Peak levels were found at a mean of 12 days after injection. Thus, in these studies, an increase in circulating VEGF was found after intramuscular injection of a plasmid expressing VEGF\(_{165}\).

Rosengart and colleagues\(^1\) have reported the effects of direct intramyocardial delivery of an adenoviral vector expressing VEGF\(_{121}\) in 21 patients. Surprisingly, a relatively large dose (up to \(10^{10}\) particle units) was associated with only a minor increase in plasma VEGF\(_{121}\) levels and only at day 3 (158 pg/mL, baseline not shown). These results are in contrast to the greater increase in VEGF\(_{165}\) after intramyocardial plasmid delivery.\(^1\) In general, in vitro and in vivo preclinical studies have shown that adenoviral vectors generally are associated with higher concentrations of local and secreted transgene products than plasmid DNA. Indeed, Guzman and colleagues\(^1\) have shown in a rat model that the intramyocardial delivery of an adenovirus vector containing the β-galactosidase gene results in significantly increased β-galactosidase enzymatic activity compared with the intramyocardial injection of β-galactosidase plasmid. Similar comparisons have been shown after intra-arterial delivery.\(^1\)

However, the apparent differences in the kinetics of transgene expression between these plasmid and adenovirus studies of VEGF expression might be explained by differences in the isoform of VEGF expressed, as well as by the form of delivery. These discrepancies illustrate once more the need for systematic assessment of the kinetics of transgene expression in humans. Furthermore, despite the growing body of knowledge on the systemic detection of transgene, these data are only surrogates for local transgene expression. Similar to the need for biodistribution data, a systematic analysis of transgene expression should be performed in all clinical studies. When possible, through either biopsy, amputation, or autopsy, target tissues should be analyzed to directly detect the kinetics and levels of local transgene expression. Furthermore, small clinical studies should be encouraged to primarily define the kinetics of transgene expression, which is a prerequisite to optimizing potential therapies while reducing toxicity, before proceeding with larger studies in which the logistics of obtaining tissue might be impossible. Defining the kinetics of transgene expression after gene transfer is a prerequisite to optimizing potential therapies while reducing toxicity.

**Restenosis Studies**

Prevention of restenosis represents a second major target for gene therapy in cardiovascular diseases. The kinetics of
Pharmacodynamics of Gene Transfer

Pharmacodynamics is defined as the study of biochemical and physiological effects of drugs and their mechanisms of action. Inherent to the field of gene transfer is the complexity of vectors both viral and nonviral, which may include biological, chemical, and physical components. Nonetheless, a clear understanding of what vectors do to patients is essential to the development of effective gene therapies. A major concern in the field of gene transfer (and a limitation to efficient transgene expression) is the immune response to vector delivery. Additionally, vectors and transgene products may have important associated toxicities and defined mechanisms of action that require definition in clinical studies.

Humoral Immune Response

Nonviral and viral vectors have the potential of inducing significant immunologic responses that may limit transgene expression, cause collateral tissue damage, or stimulate autoimmune responses. Defining the mechanisms of immunologic response and the maximum tolerated dose for each vector and delivery route should be priorities for those investigating gene transfer.

The immune response in several clinical gene transfer studies (including cardiovascular studies) has been analyzed by Harvey and colleagues. This analysis included patients receiving adenoviral vectors that continue to receive considerable attention because of their indiscriminate infectivity. In this analysis, no correlation was found between the peak serum level of antiantidenviral neutralizing antibody and the dose of adenoviral vector administered. There was a strong dependency on the route of administration, however, with the highest levels of neutralizing antibodies occurring after intratumoral delivery into liver metastases of colon carcinoma and the lowest levels occurring after intrabronchial delivery. These results are in contrast to data from laboratory animals, in which the systemic humoral reaction was dose dependent and occurred independently of the route of delivery, albeit at different peak levels, with highest titers demonstrated after intravenous administration of the viral vector. The study by Harvey et al exemplifies the fact that although some of the effects of the local or systemic administration of viral vectors can be inferred from preclinical studies, these data cannot be used in place of data from clinical studies.

A second important finding of the study by Harvey et al was the striking clinical correlation between antiantidenviral antibody levels generated and the level of preexisting antibodies directed against adenovirus serotype 5. Patients with high preexisting titers of antiantidenviral antibody mounted a greater immune response after adenovirus administration with the potential risk for reduced efficacy and increased toxicity. The relevance of these findings is suggested by preliminary data from a clinical study that used adenoviral-mediated delivery of FGF-4 in chronic myocardial ischemia. The presence of higher preexisting antibody titers to adenoviruses was associated with less improvement in exercise duration compared with patients with lower titers. Taken together, these data suggest that measurement of preexisting titers in patients receiving adenoviral vectors is essential in clinical studies and might ultimately be useful in patient selection or stratification. Furthermore, development of strategies for the use of less immunogenic viral vectors to which humans have lower preexisting titers may be important.

The other major vector used in cardiovascular gene therapy trials is plasmid DNA. Plasmids primarily have been used to induce synthesis of angiogenic factors in skeletal muscle or the myocardium, but there is a paucity of data on the pharmacodynamics of plasmid DNA in humans. In a human study of melanoma, direct delivery of DNA/liposomes failed to induce antibodies to DNA at any time. Data about the immune response to plasmid DNA in cardiovascular clinical studies are not available. Moreover, extrapolation of the lack of generation of antibodies to DNA in the intratumoral study to intramuscular and intravascular studies should be done with caution.

Vector-Associated Toxicities

In another study by Laitinen and colleagues, catheter delivery of plasmid/liposomes expressing VEGF or the reporter gene product β-galactosidase was performed in coronary arteries in patients undergoing angioplasty. There were no differences in restenosis rates, and the only laboratory abnormality was an increase in C-reactive protein in both groups. The significance and clinical relevance of this increase in C-reactive protein remain to be seen. A separate report describes the induction of lower extremity edema by gene transfer of a plasmid encoding VEGF that was thought to be related to expression of the transgene rather than to the plasmid DNA. These findings underscore the importance of defining which effects are due to the vector and which are related to the transgene product.

The effects of transgene expression may not be limited to target tissues despite local gene transfer with secreted transgene products. Concerns have been raised about the systemic effects of overexpression of angiogenic proteins. Although it has salutary effects on ischemic tissues, neangiogenesis theoretically has the potential to induce unwanted effects, such as tumoral, retinal, and plaque angiogenesis. Furthermore, secondary effects of angiogenic peptides, such as...
Defining the Mechanism of Action
Defining the mechanism of action of angiogenic peptides and antirestenotic gene transfer, as well as comparisons between transgenes and combinations, are needed.

Opportunities to Define Gene Transfer in Humans
Unfortunately, clinical trials designed to develop gene therapy products may not be ideally designed to establish the pharmacodynamics and pharmacokinetics of gene transfer. In this setting, 2 distinct opportunities exist to obtain these key data. First, these trials, often partnerships between industrial and academic investigators, should make every attempt to define biodistribution and excretion through routine analysis of bodily fluids and autopsy specimens and to obtain biopsy or other direct measures of local expression. This will require the development of assays to detect transgenes and sensitive quantitative polymerase chain reaction analysis of fluids and tissues in each study. Second, small clinical trials that attempt to directly answer questions about gene expression and function in humans should be encouraged. These studies, like that of Laitinen and colleagues\(^3\) in which clinically indicated amputation was performed after gene transfer, may advance the field in areas where standard clinical development studies might not provide these answers. Another limitation is the lack of sufficient direct comparisons between various transgenes and vectors for a certain disease (ie, FGF versus VEGF to induce angiogenesis). These limitations often derive from the increasingly important role of intellectual property and the high costs of clinical studies requiring financial support from nonacademic sources. These problems are not unique to the field of gene therapy but are no less significant. Small investigator-initiated federally funded studies may provide opportunities to compare the effects of transgenes and vectors outside of standard product development. The ultimate benefit of clinical gene therapy must be defined by randomized, double-blind clinical trials.

An important development in cardiovascular gene transfer will be the ability to noninvasively assess transgene expression. Two potential ways of doing so may be applicable to current strategies. In studies aimed to target intracellular transgenes, coexpression of a nonfunctional secreted peptide might allow for the ability to measure transgene expression indirectly, provided that there are no detrimental cellular effects of the reporter peptide (personal communication, S.J. Russell, MD, PhD, Molecular Medicine Program, Mayo Clinic and Foundation, Rochester, Minn; 2001). Although this would only be a substitute for data about local expression, it would provide for a nondestructive long-term analysis.

Noninvasive imaging of transgene expression may change the way transgene expression is defined and provide a catalyst for the entire field of investigation. Taking advantage of advances in imaging might allow for PET- or MRI-based imaging of transgene expression. An example of this is imaging of thymidine kinase gene expression with the use of labeled substrate.\(^{21,22}\) Direct imaging of light emitted by animals transduced with luciferase has been demonstrated with cooled charge-coupled device photon detection.\(^{30}\) Applications of this technology to clinical trials in cardiovascular gene transfer will greatly advance our understanding of biodistribution and the kinetics of expression.

Future Directions in Clinical Studies of Gene Transfer
Gene transfer holds great promise for the treatment of cardiovascular diseases. In this translational period of initial clinical studies, definition of the basic pharmacodynamics and pharmacokinetics of gene transfer should be included in each clinical trial. Specifically, every attempt should be made to define the biochemical and physiological effects of gene transfer vectors and their mechanisms of action. Additionally, detailed biodistribution studies with each class of vector and method of delivery should be performed, and all clinical studies should strive to obtain data to define the kinetics of transgene expression, systemic and local. Although no single trial will be sufficient, the scientific community should encourage creative ways to define these important parameters for the advancement of the field and the care of our patients.

Strong foundations are the underpinnings of great buildings and ventures. Gene transfer has extraordinary potential to radically alter disease. Although the concept is simple and elegant, the reality is that the delivery systems are extraordinarily complex. An understanding of the pharmacodynamics and pharmacokinetics of these intricate delivery symptoms is essential to provide the basic foundation for future clinical trials. In this regard, we need to know much more about the horse that will ultimately pull the cart.

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References


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