REVIEW

Gene therapy in clinical medicine

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Although the field of gene therapy has experienced significant setbacks and limited success, it is one of the most promising and active research fields in medicine. Interest in this therapeutic modality is based on the potential for treatment and cure of some of the most malignant and devastating diseases affecting humans. Over the next decade, the relevance of gene therapy to medical practices will increase and it will become important for physicians to understand the basic principles and strategies that underlie the therapeutic intervention. This report reviews the history, basic strategies, tools, and several current clinical paradigms for application.

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The potential for treating a patient using gene transfer has been realised in an exponential fashion, paralleling advances in molecular biology over the last four decades.[1–4] One of the earliest indications that gene transfer might be used therapeutically was proposed from a plant cell experiment in 1968. Rogers suggested that: "the next step was to build a modified virus… and use the virus to transmit…information".[5]

Throughout the 1970s and 1980s, basic science pushed the field of gene therapy to its first clinical trial in 1988.[6–18] However, clinical trials to date have been widely unsuccessful and have led many physicians and scientists to doubt the potential of the modality. Recent events have furthered this notion. The death of a patient involved in a University of Pennsylvania gene therapy clinical trial was a troubling development, which led to increased scrutiny of human protocols and a public backlash regarding the general safety of gene therapy.[19 20]

The "cure" of several young patients with severe combined immunodeficiency was lauded as the first significant clinical step forward for gene therapy.[21 22] However, recent reports have detailed the development of a leukaemic syndrome in several of these patients.[23] Furthermore, the leukaemia was discerned to be a direct result of the therapeutic intervention. More specifically, the viral vector produced insertional mutagenesis when incorporating into the patient's genome.[24] The development has again called into question the general safety of the current technology available for gene transfer.
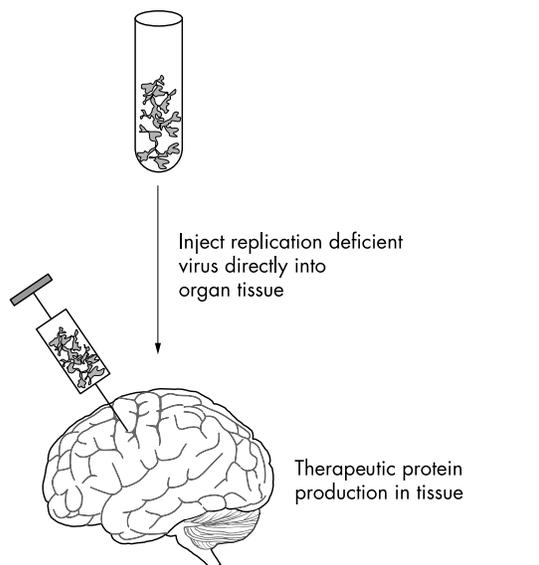
STRATEGIES FOR GENE DELIVERY
In vivo gene therapy

Two approaches for delivering genetic material exist: in vivo and ex vivo. The in vivo strategy involves the direct delivery of DNA (usually via a viral vector) to resident cells of the target tissue (fig 1). There are two requirements for such a strategy: firstly, that target cells be easily accessible for infusion or injection of virus, and secondly, that the transfer vector readily and specifically infects, integrates, and then expresses the therapeutic gene (transgene) in target cells and not surrounding cells at effective levels for extended time periods.

A discussion of all available viral vectors is beyond the scope of this review. The characteristics of the most commonly used vectors are listed in table 1. Retroviral vectors derived from the mouse Moloney leukaemia virus (MMLV) and herpes simplex virus (HSV) derived vectors are of limited value in most in vivo clinical situations.[25–27] However, in vivo application in several diseases has been established, such as HSV in peripheral neuropathies[28–30] and retroviral vectors for malignancies.[31 32] The use of non-viral, lipophilic molecules to transfer genetic material to cells in vivo has been limited by low transfer efficiency.[33]

Adenoviral vectors are the mainstay of in vivo strategies owing to their high transfection efficiency. However, the virus is plagued by antigenecity[34] and is limited to short term expression of transgenes because it does not permanently integrate into the host genome.[35] "Gutless" adenoviral vectors, which lack antigenic factors, and adenoassociated virus vectors (AAV), which have limited ability to integrate into the host genome have both been described and are under investigation as possible substitutes for the traditional adenoviral vector.[36–39]

Currently the lentivirus derived vectors appear to be the most promising for future in vivo clinical applications. The lentivirus is a species of the family Retroviridae, which can infect both non-dividing and dividing cells. Lentiviruses have evolved more complex genomes that contain regulatory genes and accessory genes in addition to the structural genes *gag*, *pol*, and *env*. These additional genes allow the virus to traverse not only the cellular, but also the nuclear membrane without prerequisite membrane disruption,[40] as is required by MMLV. This allows

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Abbreviations: AAV, adenoassociated virus; AD, Alzheimer's disease; BMD, Becker's muscular dystrophy; CABG, coronary artery bypass; CAD, coronary artery disease; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; CNS, central nervous system; DMD, Duchenne muscular dystrophy; HSV, herpes simplex virus; MI, myocardial infarction; MMLV, mouse Moloney leukaemia virus; NGF, nerve growth factor; PVD, peripheral vascular disease; shRNA, short hairpin RNA; siRNA, short interfering RNA; VEGF, vascular endothelial growth factor

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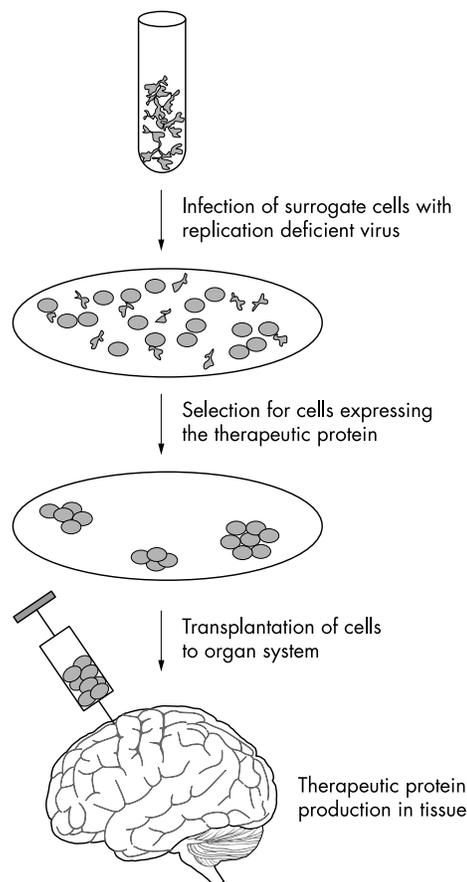
Figure 1 In vivo gene transfer. The in vivo strategy is based on the production of replication deficient virus in vitro followed by direct injection (or infusion) of the virus into an organ system. The virus genetic material will incorporate into cells and therapeutic genes will be expressed.

the virus to integrate into non-dividing cells. This factor, coupled with long term expression of transgenes and lack of antigencity, make lentiviral vectors one of the more promising new transfer methods available.

Safety issues have arisen with the proposed use of lentiviral vectors in human studies[41] because they are derived from immunodeficiency viruses, including HIV, and have the theoretical potential to generate a self replicating virus through recombinatorial events in the packaging cell line. To address this issue, the current packaging cell line requires transient transfection with three distinct plasmids, all containing gene sequences required for an active infectious virus. This system makes the possibility of three recombination events occurring in one cell to produce an actively infectious product extremely unlikely.[42–44]

Ex vivo gene therapy

The ex vivo approach involves the transfer of a therapeutic gene to cells in vitro (in culture) followed by transplantation of these modified cells to the target tissue (fig 2). The modified, transplanted cells act as an engineered secretory

Figure 2 Ex vivo gene transfer. The ex vivo strategy is based on the utilisation of a surrogate cell that is infected with virus in vitro. The surrogate cell is subsequently transferred to the target tissue and expresses the therapeutic gene.

tissue, synthesising and releasing desired proteins to the local environment.

In order to exploit the method successfully, an appropriate surrogate cell population must be identified. This cell population should be endowed with specific characteristics that fulfil several criteria. The cells should: (1) be readily available and relatively easily obtained; (2) be able to survive for long periods of time in vivo; (3) be able to express a transgene at high levels for extended durations; and (4) and not elicit a host mediated immune reaction.

Table 1 Methods of gene transfer

Method	Advantages	Disadvantages
Herpes simplex virus	Broad infectivity	Cytotoxic, low titres, and short term episomal expression
Adenovirus	High titres, infects non-dividing cells	Life threatening immunogenecity. Short term episomal expression only
Adenoassociated virus	Broad infectivity, infects non-dividing cells, non-cytopathic	Short term expression with limited integration
Lentivirus	Permanently infects non-dividing cells. High viral titres	Safety concerns owing to HIV derivation
Retrovirus	Permanent integration, well characterised	Poor infection efficiency. Does not infect non-dividing cells
Liposomes	Avoids use of cytopathic virus	Low efficiency of transfection of cells

The advantages of using an ex vivo approach include the ability to fully characterise the modified cell population before transplantation, the ability to subclone cells and produce monoclonal populations that produce high levels of therapeutic protein, and the ability to screen populations and exclude the presence of helper viruses, transformational events, or other deleterious properties obtained after or during the modification process. Furthermore, viral vectors of low transfection efficiency can be used, because uninfected cells can be selected out of the transplant population.

Multiple surrogate cells have been proposed as delivery vehicles for therapeutic genetic material (table 2). Currently, autologous primary cell cultures remain the most attractive candidate for surrogate cell delivery systems, and many experiments have demonstrated the usefulness of this cell type. Primary, adult astrocytes have been harvested and modified in vitro and have demonstrated the ability to effectively transfer genetically material to the central nervous system (CNS) for extended time periods.[45] Primary fibroblasts are an alluring surrogate cell because they can proliferate in culture, yet remain contact inhibited and non-transformed, even after multiple passages in vitro. Furthermore, these cells can be easily harvested from the host, allowing for autologous cell transplantation. Many studies have demonstrated the utility of using primary fibroblasts for gene transfer,[46 47] and a current clinic trial is underway using these cells in an ex vivo strategy to treat Alzheimer's disease.[48] The advantage of using primary, autologous cell cultures include the lack of antigenicity and decreased risk of malignant transformation relative to immortalised cell lines. Disadvantages include difficulty in harvesting some types of primary cells, maintaining them in culture, and effectively expressing transgenes through current transfection techniques. Another complication arises when primary cells are transferred to non-host tissue; for example, primary fibroblasts transplanted to the CNS will often produce collagen and other skin appropriate products that interfere with normal CNS functioning.[49] This problem may be overcome with the use of stem cells.

Adult stem cells have become a viable option for gene transfer over the past decade and are similar to primary cell cultures. However, adult stem cells offer the potential to completely incorporate into any host tissue and transform into a mature cell of that organ. This ability ensures long term survival of grafted cells, which function in concert with the resident cells of that organ system. Peripheral derived haematopoietic stem cells are of particular interest as a potential surrogate cell. The plasticity of this cell type has been widely reported; bone marrow derived glial cells have been identified in focal ischaemic rat brain,[50] and bone marrow derived myocardial cells have been identified in rat models of cardiac ischaemia.[51 52] This underlines the major advantage of stem cells over primary cells; the possibility that these cells could be used not only to carry therapeutic proteins, but also to repopulate organs with damaged or depleted cell numbers. Haemopoietic stem cells are easily obtained through basic peripheral intravenous access systems, allowing for marrow derived stem cells to be harvested systemically, modified in vitro, and under the correct circumstances re-infused into the peripheral blood with subsequent homing to damaged target tissue such as brain or myocardium.

Bone marrow derived stem cell use has been limited by low viral transfection efficiency[53 54] and technical difficulties in isolating, culturing, and maintaining these cells. Other adult stem cells include hepatocytes, which have been obtained through partial liver transections, and have been isolated, manipulated in culture, and then re-infused into autologous liver.[55] CNS stem cells have also been isolated but are not available for autologous transplant owing to the inaccessibility of these cells in the periventricular zone of the CNS. Most studies have used cadaver derived cells.[56 57]

Finally, fetal derived stem cells have been the topic of much scientific and media speculation. Fetal cell transplantation has been successful in multiple animal models of disease,[58–62] and transfer of unmodified fetal cells has already been undertaken in Parkinson's disease.[63–65] Patients with Parkinson's disease receiving fetal dopaminergic neurones have demonstrated clinically significant long term benefits. However, several patients obtained no benefit from the transplant and several developed worsening of symptoms.[66–68] Similarly, transplantation of human fetal striatal tissue to patients with Huntington's disease has been undertaken. Results indicate that grafts derived from human fetal striatal tissue can survive regardless of the ongoing endogenous neurodegenerative process; however, the clinical benefit is unproven.[69–71] Finally, fetal islet cells have been transplanted to patients with diabetes mellitus with variable success. In some cases the transplant has obviated the need for exogenous administration of insulin.[72 73] The fact that fetal cells can be maintained in culture, have some degree of plasticity, and can be transfected using classical methods make this cell type attractive. However, fetal derived primary cell cultures are often heterogeneous and difficult to define and purify. The success of graft survival appears to be related to the manner in which cells are prepared, purified, and transplanted, but the factors predicting a successful clinical response to graft transplantation are unclear. Further, fetal tissue is not readily accessible and continues to be part of a wider moral and ethical debate.

Table 2 Surrogate cell types

Cell type	Advantages	Disadvantages
Transformed cell lines	Easily obtained, cultured and manipulated. Survive after transplant	High degree of malignant transformation of cells after transplant. Unsafe
Primary cell cultures	Decreased risk of transformation. Relatively easy to infect	Can be difficult to obtain or culture. May not survive long term after transplant
Adult stem cells	Plasticity of cell type with ability to potentially incorporate into target organ tissue	Difficult to maintain in culture and to infect while maintaining stem cell status
Fetal stem cells	Plasticity of cell type has potential for incorporation and regenerating target tissue	Often heterogeneous and difficult to purify. Moral and ethical problems remain a major impedence to availability. Heterologous transplant only

Table 3 Clinical trials

Trial	Gene expression	Clinical efficacy
CF		
Aerosolised AAV-CFTR phase I[81]	Not detected	Not reported
Maxillary sinus AAV-CFTR phase I[82]	Not detected	No improvement
Aerosolised rAAV2-CFTR phase II[83]	Limited expression with low copy number/cell	Awaiting phase IIb results
DMD		
Phase I DMD naked plasmid DNA-dystrophin[94 95]	Results pending	Results pending
Alzheimer's disease		
Ex vivo NGF/fibroblasts phase I[48]	Results pending	Results pending
Cancer		
P53 replacement AV-p53 recurrent glioma phase I[145]	Limited to the injection site	Modest survival benefit
Phase II/III ovarian cancer AV-p53 replacement[120]	Not determined	No improvement
Phase I non-small cell lung AV-p53 replacement[116]	Not determined	Suggested survival benefit
HSV-TK phase I mesothelioma[125 126]	Not determined	No improvement
HSV-TK phase I refractotory brain cancer[127–129]	Not determined	No improvement
CAD/PVD		
BIOBYPASS phase I CAD[139]	Not determined	Increased exercise tolerance and increased myocardial perfusion
BIOBYPASS phase I PVD[135]	Not determined	Increased exercise tolerance and increased ankle: brachial index. Improved quality of life

PVD, peripheral vascular disease; CAD, coronary artery disease; AV, adenoviral vector; AAV, adenoassociated viral vector; NGF, nerve growth factor; DMD, Duchenne muscular dystrophy; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator.

GENE THERAPY APPLIED TO CLINICAL MEDICINE (TABLE 3)
Diseases caused by single gene mutations
Diseases caused by a single defective gene represented an early target for corrective gene therapy. Diseases such as Duchenne muscular dystrophy (DMD) and cystic fibrosis (CF) have well established aetiologies and pathophysiologies, with clearly defined genetic mutations.

Cystic fibrosis
CF is caused by mutations in a gene on chromosome 7, named cystic fibrosis transmembrane regulator (*CFTR*). This 230 kb gene encodes a 1480 amino acid protein that acts as a membrane chloride channel.[74 75] As many as six different mutation types and 1000 specific mutations have been identified, and vary in frequency worldwide.[76] The defect results in changes in multiple organ systems, most notably the lungs and pancreas, producing chronic lung infection, pancreatic insufficiency, and diabetes mellitus. The median survival in 2000 was 32 years.[77]

Restoration of the wild type *CFTR* could be curative. The first phase I clinical trials ever conducted using adenoviral vectors and AAV vectors involved the transfer of *CFTR* to CF patients.[78 79] Trials using first and second generation adenoviral vectors have been limited by the inability to repeatedly administer the virus. The transient nature of the viral expression vector requires such a strategy, but the inflammation induced by these vectors prevented this. Early trials using AAV did not induce inflammation, but failed to demonstrate effective levels of transferred CFTR expression.[79 80] Target Genetics Corporation developed an adenoassociated virus tgAAVCF virus, expressing the *CFTR* gene, and has administered it to patients in an aerosolised form. Results showed that a single administration of the virus was well tolerated and safe, but virus derived CFTR expression was not detected in patients. Clinical efficacy was not reported.[81] Phase II trials have also been reported using the same vector delivered to the maxillary sinuses of CF patients. Results confirmed the safety of tgAAVCF administration, but again failed to detect expression of the transferred *CFTR* gene in biopsy specimens and failed to demonstrate clinical improvement in treated patients.[82] A phase I trial has recently been published using a second generation AAV (rAAV2) expressing CFTR. Result indicated that a single administration of the virus was safe with escalating virus concentration; however, the number of cells in the airway expressing the viral CFTR were limited and they contained a low copy number. Both results indicate inefficient transfer of genetic material using this virus.[83] A phase IIb trial is underway to determine if the therapy improves lung function in CF patients.[84]

Similarly, early clinical trials using several different cationic lipid preparations were deemed safe and allowed for repeated administration of *CFTR*, but were inefficient in transferring the gene, and failed to demonstrate efficacy.[85–88]

Finally, a clinical trial is being undertaken by Copernicus Therapeutics Inc, using a novel method that allows for compaction of a single molecule of DNA condensed to the minimal possible volume. The small volume, positively charged particle is able to pass through cellular and nuclear membrane pores and allows delivery of genetic material to non-dividing cells.[89] Transfer of genes using this technology has proven to be safe in animals, and subsequent phase I clinical trials in CF patients have been completed.[90] In the study, patients received compacted DNA containing *CFTR* via the nasal passages. Results indicated that the administration is safe and tolerable. The treatment efficacy is not noted in the phase I trial. A phase II, multicentre, double blind, placebo controlled study is underway.[91]

Duchenne muscular dystrophy

DMD, the most prevalent muscular dystrophy, is caused by large deletions or insertions of the dystrophin gene. This very large gene (cDNA 14 kb) encodes a 3685 amino acid protein[92] that stabilises the muscle cell membrane. Its dysfunction results in destabilisation and subsequent degeneration of muscle tissue.[93]

As with CF, DMD is potentially curable with extensive transfer of the wild type dystrophin gene to muscle tissue. In a similar fashion, strategies are limited due to the large size of the dystrophin gene. Currently, a phase I trial has been initiated using plasmid dystrophin DNA. The naked plasmid is directly injected into the radial muscle in an attempt to determine tolerability and safety as well as gene expression.[94 95] Results have yet to be published. More promising clinical trials should be undertaken using viral vectors to produce "exon skipping" of mutated sequences of the dystrophin gene (discussed later).

Other diseases
Alzheimer's disease

Alzheimer's disease (AD) is the most common cause of dementia. AD generally affects individuals in their sixth and seventh decade, and is characterised by the insidious onset of cognitive changes manifesting initially as forgetfulness. Once the memory defect is established, defects in other areas of cerebral function become obvious, leading to behavioural disturbances including anxiety, agitation, and confusion. The progressive decline in cognitive function usually occurs over five to 10 years, ending with the patient bedridden and succumbing to pneumonia or other recurrent infection.

The disease course is tragic not only for the patient, but for the family and caregivers. It is estimated that four million Americans suffer from the disease, with an average yearly cost to the USA of $100 billion.[96] Current understanding of the pathophysiology is limited. Current treatment, consisting of acetylcholinesterase inhibitors, modestly retards symptomatic disease progression, but does not prevent neurone loss.[97–99]

However, basic research into neuroprotective factors (factors that prevent neuronal death) over the past two decades has now culminated in a phase I, gene therapy clinical trial for the treatment of AD. The neuroprotective factor (neurotrophic factor) being utilised in the study is nerve growth factor (NGF). NGF was initially described in the 1950s after it was extracted from chick embryos and shown to promote neuronal survival.[100] In the 1980s, several groups reported that NGF administration into brain parenchyma prevented the loss of cholinergic neurones in the forebrain following axotomy,[101–103] and subsequently, NGF was shown to have an effect on age related CNS decline in normal rats. Initially, therapeutic methods focused on intracerebroventricular infusions of NGF to rescue dying neurones; however, several groups reported significant toxic side effects in mice. Successive observations in the early 1990s showed that this method of NGF delivery caused weight loss, decreased appetite, and hypertrophy of cells in the subpial space.[104–106] Those animal studies foreshadowed the fate of an intracerebroventricular NGF infusion clinical trial, as three patients with AD receiving infusions developed adverse effects, leading to its premature cancellation.[107]

It became evident that an alternative delivery method was required if NGF was to be used therapeutically. Following multiple rat and primate gene therapy studies, including dose escalation studies,[48] demonstrating the effectiveness and safety of NGF gene therapy in preventing neuronal loss and the absence of significant side effects,[108 109] an ambitious, phase I, ex vivo gene therapy clinical trial was initiated in 2000. Primary autologous fibroblasts were infected, in vitro, with an MMLV derived viral vector that expressed NGF constitively from a viral promoter sequence. These infected cells, expressing high levels of NGF, were then transplanted into the nucleus basillis of human subjects.

At the last published report the group had enrolled eight individuals in the phase I safety trial. All patients enrolled were early stage, with "probable" AD based on strict clinical research criteria. The study will terminate when all patients have been studied for one year post-transplantation. The goal of the study is to determine the effect of ex vivo gene therapy strategies for CNS disease and to determine if NGF can prevent clinical decline.[48]

Cancer

The second leading cause of death in the USA is cancer, with cancer deaths approaching 500 000 annually, and one million cases of cancer diagnosed each year.[110] Current methods of treatment, including chemotherapy, radiation therapy, and surgical debulking, are generally only effective for early stage disease. The more advanced the disease, the less effective the therapy becomes. Furthermore, the side effect profile of chemotherapy is horrifying and many treatment failures are due to intolerable side effects and the inability to continue with an entire treatment course. Many gene therapy clinical trials have been initiated since 1988 to treat cancer. Although multiple studies have been initiated and strategies proposed, only two broad strategies are discussed here.

Replacement gene therapy using *p53* is based on the broad concept that correction of a specific genetic defect in tumour cells can reverse uncontrolled cell growth. The wildtype p53 gene product is involved in the recognition of DNA damage and the subsequent correction of that defect or induction of apoptosis in that cell.[111–113] The gene is altered in over 50% of human malignancies,[114] and therefore, has become the fulcrum of multiple gene therapy replacement trials. The general strategy is an in vivo gene therapy approach using an adenoviral vector expressing the wildtype *p53* gene. The adenovirus delivery mechanism varies depending on where the tumour is located, and in all studies the therapy is combined with surgery, radiation, or chemotherapy, or a combination of the three. Clinical trials in various phases are underway for treatment of glioma,[115] lung cancer,[116] ovarian cancer,[117] breast cancer,[118] and recurrent head and neck cancer.[119] Results published to date have been disappointing. Phase I trials for recurrent glioma reported only modest survival benefit and expression of adenoviral derived p53 only a short distance from the site of virus administration.[115] Phase II/III trials for ovarian cancer failed to show treatment benefit with intraperitoneal administration of adenovirus expressing p53 with chemotherapy after debulking surgery.[120] Finally, Swisher *et al* published antitumour effects associated with the treatment of non-small cell lung cancer; however, no comparable control group was described in their report.[116]

A more elegant strategy for the treatment of cancer involves the use of the HSV thymidine kinase gene (*HSV-tk*) and the prodrug gancyclovir. Gancyclovir is used clinically as an antiviral agent against HSV, Epstein-Barr virus, and cytomegalovirus infection.[121] Cells infected with these viruses produce a thymidine kinase that catalyses the conversion of gancyclovir to its active triphosphate form. The triphosphate form is incorporated into DNA and results in chain elongation termination, leading to the death of the cell.[122] The concept of "suicide gene therapy" was initially described in the late 1980s, based on prodrug activation.[123 124] It was proposed that cancer cells be infected with a virus expressing HSV-tk, resulting in constituent expression of the drug activating enzyme in these cells. Subsequent exposure of these infected cancer cells to gancyclovir results in drug activation and death of malignant cells (fig 3).
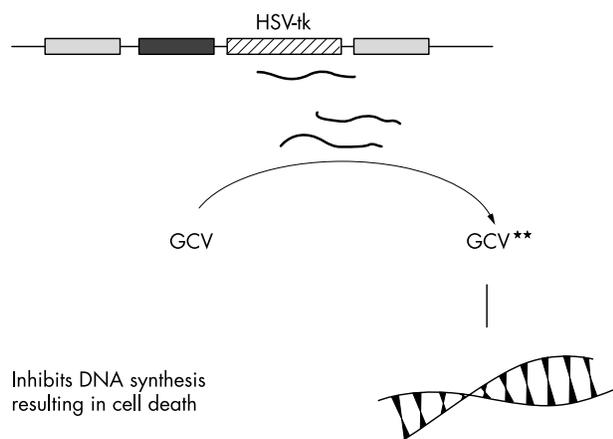
Figure 3 Suicide gene therapy. A virus expressing the drug activating enzyme, HSV-tk, is used to infect malignant cells. Malignant cells are then exposed to gancyclovir, which is activated to a cell lethal form by the HSV-tk enzyme. Cells not infected with the virus, and thus, not expressing HSV-tk, are not affected by the administration of gancyclovir. GCV, gancyclovir; GCV**, activated gancyclovir.

Multiple clinical trials have been undertaken utilising the suicide gene therapy study. In 1998, a report of 21 patients with mesothelioma was published. Patients received intrapleural injection of adenovirus expressing HSV-tk followed by gancyclovir exposure. Multiple toxicity issues were reported with this study without clinical benefit being noted.[125] In another trial, 18 patients with prostate adenocarcinoma were injected with adenovirus expressing HSV-tk followed by gancyclovir exposure. Multiple adverse effects were again noted, and only three patients experienced transient tumour regression.[126]

In 1997, Ram et al reported the treatment of refractory recurrent brain malignancy with suicide gene therapy.[127] Survival benefit was not appreciated in the treated group. In a multinational study, 48 patients with recurrent gliablastoma multiforme received HSV-tk/adenovirus injections into the wall of the tumour cavity after resection, with subsequent gancyclovir exposure for 14 days. No clinical benefit was noted.[128] A third trial, accessing the use of HSV-tk/gancyclovir therapy for patients with recurrent primary or metastatic brain tumours, was undertaken and also failed to demonstrate significant clinical benefit.[129]

Atherosclerosis

Atherosclerosis remains one of the greatest health problems for developed countries. About 12 million Americans have coronary artery disease (CAD) and 1.1 million have a new myocardial infarction (MI) each year.[130][131] Advances have been made in the treatment of atherosclerosis, acute MI, and peripheral vascular disease (PVD); however, morbidity and mortality remain significant, as do treatment failures and rate of recurrence.

The value of collateral blood vessel supply to ischaemic tissue has been recognised for some time. Specifically, the extent of collateral blood vessel supply is inversely proportional to the extent of tissue damage incurred during an acute MI. Individuals lacking significant collaterals who suffer an acute event have more tissue damage and subsequent greater impairment of end organ function. Thus, collateral circulation is a natural "bypass" system, which is slow in developing, but effective at decreasing damage to tissue supplied by a diseased artery.

Consequently, it was proposed that the "bio-induction" of more effective collateral circulation could be used in patients who were not candidates for traditional surgical procedures

and were failing medical management, or possibly to replace traditional forms of coronary artery bypass grafts (CABG) or stents.

Vascular endothelial growth factor (VEGF) became the leading candidate molecule for the induction of angiogenesis.[132] However, the short half life of VEGF (about seven minutes) and the extended exposure time required to induce effective angiogenesis in animal models[133][134] was not compatible with protein infusion or injection techniques. Gene therapy was proposed as the appropriate form of delivery as it would allow for sustained, local protein delivery to ischaemic tissue over several weeks. The specific in vivo gene therapy strategy proposed was called "BioBypass". It consisted of an adenovirus modified to express the VEGF cDNA sequence, which was then injected into ischaemic tissue. Cells infected would express VEGF and induce angiogenesis into the region of ischaemia. The transient nature of adenovirus expression in cells allowed for continuous expression of VEGF for about four weeks.[135] This time frame is long enough for angiogenesis, but short enough to limit potential side effects of persistent growth factor expression, including malignancy.

Toxicology studies in animals have demonstrated the safety and tolerability of BioBypass,[136][137] and therefore the concept has proceeded to clinical trials. Phase I clinical trials of 33 patients with limb ischaemia revealed improvement in exercise tolerability, increase ankle: brachial index, and quality of life after 20 injections of the adenovirus expressing VEGF into the affected ischaemic limb tissue.[138] Unfortunately, more rigorous phase II trial results have recently been published and are disappointing. Results indicate that BioBypass did not effectively relieve ischaemia in patients with PVD after virus injection.[139]

Two related phase I trials have been conducted in patients with CAD. One study combined the intramyocardial injection of adenovirus expressing VEGF with concurrent CABG. The second study was conducted on non-surgical candidates failing maximum medical management, who received intramyocardial injection of virus though microscopic thoracotomy. Results indicated an increase in myocardial tissue perfusion and an increase in exercise tolerance after treatment.[140] Further trials are pending.

LIMITATIONS

General limitations of current gene therapy technology include inefficient gene transfer. Viral vectors are extremely inefficient at transferring genetic material to human cells; even those with very high transduction efficiency in vitro fail to produce significant infection rates when applied to clinical trials. This factor played an important role in the escalation of dose in the ill fated University of Pennsylvania gene therapy clinical trial.

Another overarching issue is the lack of viral specificity. Current techniques do not allow for specific infection of cells; rather, cells in the vicinity of virus delivery are randomly infected. This issue has been partially addressed by the use of tissue specific promoters, which allow expression of the transgene only in tissue that can activate a specific promoter. However, this strategy is not amenable to all disease states and it continues to suffer from technical difficulties such as promoter "leakage" from endogenous viral sequences.

Another issue is the lack of long term transgene expression. Although not a concern in some clinical settings, as was demonstrated in PVD and CAD gene therapy, the need for long term expression of a therapeutic gene is essential in other strategies. For example, if the patients in the AD trial received fibroblasts that only expressed neurotrophic factor and salvaged neurones for one year, would the risk of the procedure outweigh the benefit considering that current

medication also delays progression for approximately one year? The problem has been noted in multiple in vitro studies and animal studies where initial high levels of therapeutic protein have resulted in clinical responses, only to be lost several months later.[141]

A final issue, and perhaps most important, is that of controlled gene expression. The ability to turn ''on'' and ''off'' the expression of a therapeutic gene will be essential for those strategies requiring long term expression and those inducing inflammation or utilising growth factors. Induction of inflammation for treating such diseases as cancer may be useful, but once the cancer is cured the inflammation continues if bystander cells are expressing the inciting transgene. Chronic inflammation of a specific tissue is undesirable. Similarly, with the use of growth factors, uncontrolled growth factor expression and function is intimately involved in the malignant transformation processes. The continual expression of a growth factor predisposes to malignancy, as was noted by the researchers conducting the AD clinical trials.[48] It is essential to be able to turn off growth factor expression if malignancy is detected, or if treatment is toxic or no longer deemed useful or necessary. To this end, progress has been made in the development of inducible promoter systems, for example, a tetracycline inducible promoter system has been defined in which the presence of tetracycline (which can be taken orally by patients) will allow the activation of a promoter sequence and result in subsequent therapeutic gene expression. In the absence of tetracycline, theoretically, the transgene is not expressed (fig 4). This ''on/off'' system allows for important dose delivery control. However, these systems are generally plagued by ''leakage'' of promoter activity and are currently imperfect.[142] [143]

SAFETY ISSUES
Since the approval of the first clinical gene therapy trial in 1988 and its commencement in 1989, over 3000 patients have been treated with gene therapy. Many of the initial safety considerations raised with early trials remain today. These can be broadly categorised as either pertaining to the delivery vector or the expression of the transferred gene.

The vast majority of clinical trials exploit viruses to transfer expression of genetic material to cells. Administration of a virus can result in inflammation or active infection. The risk
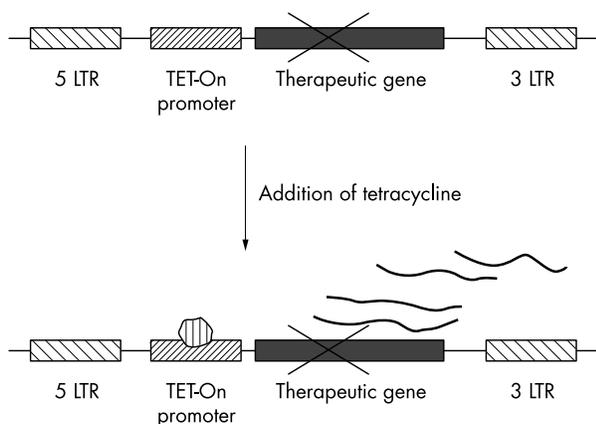
Figure 4 Controlled gene expression. The Tet-On system is an example of controlled gene expression. In the absence of tetracycline, the promoter is not activated and therefore therapeutic gene expression does not occur. However, when tetracycline is given to the patient the promoter sequence is activated and the therapeutic gene expressed; the wavy lines indicate gene transcription and translation into active protein.

of overwhelming inflammation from virus administration was experienced firsthand during the University of Pennsylvania study, which resulted in the death of an 18 year old participant.[20] Secondly, active uncontrolled infection can occur either through multiple recombination events (unlikely given the current design) or through the contamination of replication incompetent viral stocks with a helper virus. There are no known cases of contaminated virus being delivered to patients, and clearly, the testing of material destined for clinical trials is essential and quite routine. Thirdly, the administration of retrovirus, which incorporates randomly into the genome, can result in insertional mutagenesis and malignant transformation. As mentioned previously, the treatment of severe combined immunodeficiency with autologous transplants of stem cells infected with a retrovirus expressing the deficient gene resulted in the alleviation of symptoms in patients. However, two of the 11 patients developed T cell leukaemia because the virus integrated downstream from an oncogene, resulting in its upregulation.[21–24]

The expression of various types of therapeutic genes predisposes patients to adverse effects. As mentioned earlier, the utilisation of growth factors for neurodegenerative disease or the use of proangiogenic molecules for CAD can promote tumour growth. Likewise, the expression of proinflammatory cytokines for the treatment of malignancy can result in aberrant inflammatory conditions. Although the administration of any therapeutic agent is associated with side effects, the complete inability to withdraw the agent delivered via gene therapy is particularly troublesome.

Finally, there is a theoretical risk of inadvertent alteration of germline cells. The event has been reported in animal models, but is yet to be accurately described after administration to humans.

FUTURE DIRECTIONS
New strategies for the application of gene transfer to the treatment of human disease are rapidly evolving. Two emerging techniques, RNA interference and exon skipping, hold particular promise.

RNA interference, or post-transcriptional gene silencing, was initially demonstrated in invertebrates and plants.[144] The technique results in the selective and persistent suppression of gene expression and has been used extensively to study gene function. Briefly, double stranded RNA, homologous to the gene targeted for suppression, is introduced into cells where it is cleaved into small fragments of double stranded RNA named short interfering RNAs (siRNA). These siRNAs guide the enzymatic destruction of the homologous, endogenous RNA, preventing translation to active protein.[145–148] They also prime RNA polymerase to synthesis more siRNA, perpetuating the process, and resulting in persistent gene suppression.[149] [150]

To effectively silence specific genes in mammalian cells, Elbashu *et al* designed short hairpin RNA (shRNA). These sequences, which can be cloned into expression vectors and transferred to cells, result in the transcription of a double stranded RNA brought together by a hairpin loop structure (fig 5). These shRNAs effectively mimic siRNA and result in specific and persistent gene suppression in mammalian cells.[151] Multiple groups have effectively incorporated shRNA coding sequences into AAV and lentiviral vectors and demonstrated specific gene suppression in mammalian cells.[152–155]

This technique has possible application to various disease states, including HIV infection. The suppression of specific viral protein expression could prevent viral replication and propagation. The technique could also suppress expression of
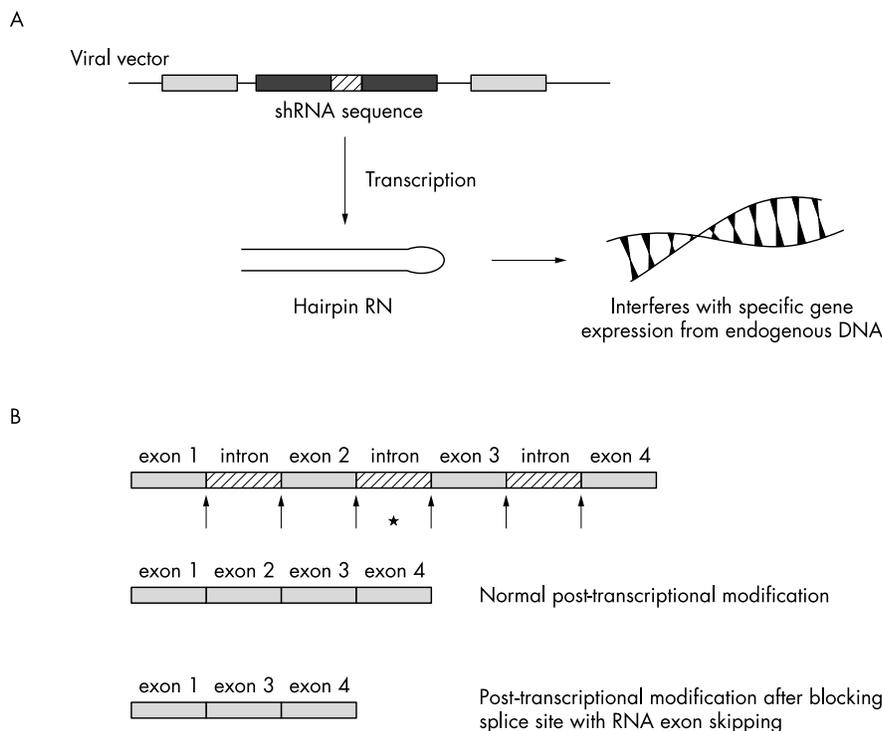
Figure 5 RNA interference and exon skipping. (A) RNA interference silences specific gene expression. The ''turning off'' of a gene is mediated by the expression of a short homologous RNA sequence from a viral vector that forms a hairpin. (B) Exon skipping is mediated by the expression of a short fragment of RNA that binds to a splice site (arrows). This prevents normal post-transcriptional modification and results in a protein with one fewer exon. In this example, the splice site before exon 2 is blocked (*) and exon 2 is spliced with the introns from the final transcript.

aberrant gene products or inflammatory proteins that may contribute to disease states.

A second emerging technique, exon skipping, may have important therapeutic implications for patients with DMD. The technique uses short sequences of RNA that are complementary to exon recognition sequences or exon splicing enhancer sequences. The expressed complementary RNA will bind to these regions of the gene and prevent the splicing of intron and exon at that site.[156–158] The result of the altered post-transcriptional processing is the removal of a target exon from the final protein (fig 5).

This technique is particularly relevant to DMD. DMD results from frameshift mutations (reading frame is not preserved) that produce a completely aberrant protein. However, the less severe phenotype, Becker's muscular dystrophy (BMD), results from an in frame mutation (reading frame is preserved) in the same gene. Thus, an exon with an insertion or deletion that disrupts the reading frame of the dystrophin gene (producing DMD) could be spliced out using the exon skipping technique. The reading frame would be maintained and the dystrophin gene would be converted from a frame shift mutation to an in frame mutation. More importantly, a patient with DMD could be converted to a BMD phenotype, resulting in decreased morbidity and prolonged life expectancy.[159 160]

CONCLUSION

In conclusion, gene therapy continues to offer great promise in treating disease for which ineffective modalities exist or no options are available. The basic paradigm for gene delivery has been constant for some time, in vivo and ex vivo. However, the strategies to deliver genetic material within these paradigms are highly variable and new, insightful techniques emerge continuously. Currently gene therapy based clinic trials are being conducted in many of the major disease processes and in rare, usually well defined, genetic disorders. As basic science continues to progress within the field, more effective clinical trials will be launched and eventually will successfully treat or even cure patients. As this occurs, it will be essential for all physicians to understand the basic concepts encompassing a complex field.

APPENDIX 1: GLOSSARY OF TERMS

Autologous: Donor cells and recipient organism are the same; in contrast to heterologous in which cells and recipient are genetically different.

Antigenicity: The potential to act as an antigen and induce an inflammatory immune response.

Cell culture: Refers to growing cells in an artificial environment, using a flask, synthesised medium, and an incubator.

Copy number: The number of individual genes that were transferred to a cell. Generally, this level correlates with level of gene product.

Exon skipping: A technique that allows selected exons to be deleted from the final protein. The process is mediated by transcription of sequences homologous to exon splice sites.

Expression: Refers to the transcription and translation of a bioactive protein in a cell.

Ex vivo gene therapy: A two step strategy to transfer genetic material to an organ system. The first step involves the transfer of the gene to a surrogate cell. The second step involves the transfer of the surrogate cell, expressing a therapeutic protein, to an organ system.

Frame shift mutation: A mutation that disrupts the RNA reading frame, resulting in a completely abnormal amino acid protein sequence after the mutation. In contrast, ''in frame'' mutations preserve the original reading frame, and therefore, after the mutation the amino acid sequence of the protein is unchanged from the wild type protein.

Helper virus: A virus (usually contaminant) that aids in the replication of a virus that by itself cannot replicate.

Integrate: The permanent incorporation of a DNA sequence into the genome of a cell, resulting in its persistence after cell division.

In vitro: Refers to the artificial environment of culture flasks and supplemented medium.

In vivo: Refers to the natural environment.

In vivo gene therapy: A strategy to deliver genetic material by directly administering the transfer vector to target cells.

Monoclonal: A population of cells derived from one cell; these cells should be genetically identical.

Packaging cell line: A cell line that is engineered to produce virus particles.

Polyclonal: A population of cells derived from multiple cells. These cells are genetically and phenotypically heterogenic.

Post-transcriptional gene silencing (RNA interference): A technique used to suppress the expression of an endogenous gene. The process is mediated by the expression of short pieces of RNA that are homologous to the gene targeted for suppression.

Replication incompetent virus: A virus that is limited to one round of infection.

Self replicating virus (replication competent): A virus that can infect cells and subsequently produce infectious progeny to infect further cells.

shRNA (short hairpin RNA): Short RNA sequences that form a hairpin structure after transcription. The shRNA will interfere with endogenous gene expression in mammalian cells and is homologous in function to siRNA.

siRNA (short interfering RNA): Short pieces of RNA that bind to endogenous RNA sequences and mediate their degradation, preventing translation to a protein.

Subclone: A monoclonal population isolated from a polyclonal population.

Surrogate cell: A cell type used to transfer expression of a therapeutic protein to an organ system.

Therapeutic gene: A gene transferred to a cell, which when expressed will hypothetically alter disease course favourably.

Transgene: Synomonous with ''gene of interest'' and ''therapeutic gene''.

Viral vector: Altered virus that has been engineered to contain one or several genes of interest in addition to viral sequences that code for transcriptional and packaging signals and viral structural proteins. This structure ''carries'' the therapeutic gene to cells so that it can be expressed.

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