Gene therapy—perspectives and promises

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Gene therapy is a novel and promising treatment for inherited and acquired genetic disorders. Basic laboratory investigations and clinical trials have shown that gene therapy in vivo is feasible, results in biological responses, and is relevant to different types of human diseases. To achieve clinical efficacy, current research has focused on improving the efficiency and specificity of gene transfer. Furthermore, gene therapy unlocks new research approaches and offers important insights into disease mechanisms. The emphasis of gene therapy has also shifted from the treatment of inherited genetic diseases to cancer. Despite the significant progress made in our understanding of gene therapy, considerable conceptual and practical limitations still exist. Evidence of the efficacy of gene therapy, however, is compelling. Many of the current problems of gene therapy should be overcome and gene therapy will become an important treatment modality for genetic diseases.

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Introduction

Recent advances in cellular and molecular biology have greatly increased our understanding of the genetic bases of diseases. This forms the conceptual and technological foundation for the development of gene therapy.[1] Gene therapy can be defined as the transfer of genes to the cells of an individual, resulting in therapeutic benefit.[2] The transfer of genes can be carried out ex vivo, whereby genes are inserted into cells in the laboratory. Alternatively, it can be performed in vivo, in which genes are directly transferred into cells within an individual. Many means are available for gene transfer, and feasibility and logistics are often the factors governing the methods adopted.

Conceptual aspects of gene therapy

The nature of disease and the target cells involved

Gene therapy is directed at correcting genetic defects in a cell. These genetic defects can be inherited or acquired. With inherited genetic defects, the clinical manifestations depend on the cell types in which the gene of interest is expressed. For example, in phenylketonuria, the gene coding for phenylalanine hydoxylase is mutated. As the gene is normally expressed in the liver, gene therapy directed at insertion of the wild type gene into liver cells will be adequate in correcting the abnormal phenotype.[3] Similarly, in thalassaemia, the delivery of wild type globin genes into haematopoietic stem cells will be appropriate.[4] On the other hand, if the genetic defect involves a circulating molecule, such as the coagulation factors VIII or IX, all that is necessary may be the transfer of the respective genes into cells that will secrete them, instead of specifically into liver cells.[5-7]

The most commonly acquired genetic diseases are cancers. In localised tumours, delivery of the therapeutic gene locally may suffice. However, if the cancer is disseminated, strategies that involve systemic gene delivery are required.[8]

Regulation of the transferred gene

The expression of genes is tightly controlled in the body. During development, genes are turned on and off according to a well co-ordinated schedule. In adult

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life, genes are regulated both by genetic and physiological factors. While under-expression of the transferred gene will decrease its efficacy, over-expression of it may result in toxicity. Gene regulation is particularly important in the treatment of inherited disorders, where the therapeutic goal is prolonged gene expression at physiological levels.

Persistence of the transferred gene

In inherited germline genetic diseases, gene expression on a long term basis is required. Permanent integration of the transferred gene into the target cell genome is therefore a distinct advantage. On the other hand, for treatment of somatic genetic diseases such as cancer, where the aim is eradication of the transduced cancer cells within a short time, transient gene expression is desirable and prolonged expression may actually be detrimental.[9]

Methods of gene transfer

Construction of an expression cassette

For a gene to be expressed, two DNA sequences are essential—the promoter and the polyadenylation signal. The promoter is the region where RNA polymerase binds to initiate transcription, while the polyadenylation signal encodes an RNA sequence that provides the site for addition of a multiple adenosine sequence necessary for mRNA processing.[10] When a therapeutic gene, which can be genomic DNA or cDNA, is placed between a 5′ promoter and a 3′ polyadenylation signal, an expression cassette is formed (Fig 1).

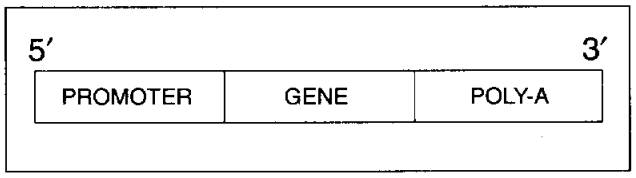

Fig 1. Structure of an expression cassette. Poly-A polyadenylation signal

Vehicles for gene transfer

Isolated DNA molecules are stable in vitro but entry into cells occurs at a very low efficiency, even with the help of physiochemical methods. However, DNA is unstable in vivo, so that a large amount is required for direct application. To facilitate efficient gene transfer, vectors are required. Vectors can be viral or non-viral, and they transfer expression cassettes through different mechanisms and thus have respective advantages or disadvantages (Table 1).

Retroviral vectors

The mechanism of gene transfer of a recombinant replication-deficient retroviral vector is illustrated in Figure 2.[11] Retroviruses transfer the therapeutic gene into the host cell DNA through reverse transcription and integration, so that the transferred gene is permanently incorporated into the host genome. This is an advantage in the treatment of hereditary diseases, although it carries the risk of insertional mutagenesis (Table 1). The use of retroviral vectors in vivo is limited by the sensitivity of the vector to inactivation, the requirement of dividing cells, and the low titres (typically 10^4-10^6 plaque-forming units [pfu/mL]). For these reasons, retroviral vectors have been used almost exclusively in ex vivo gene transfers.[11]

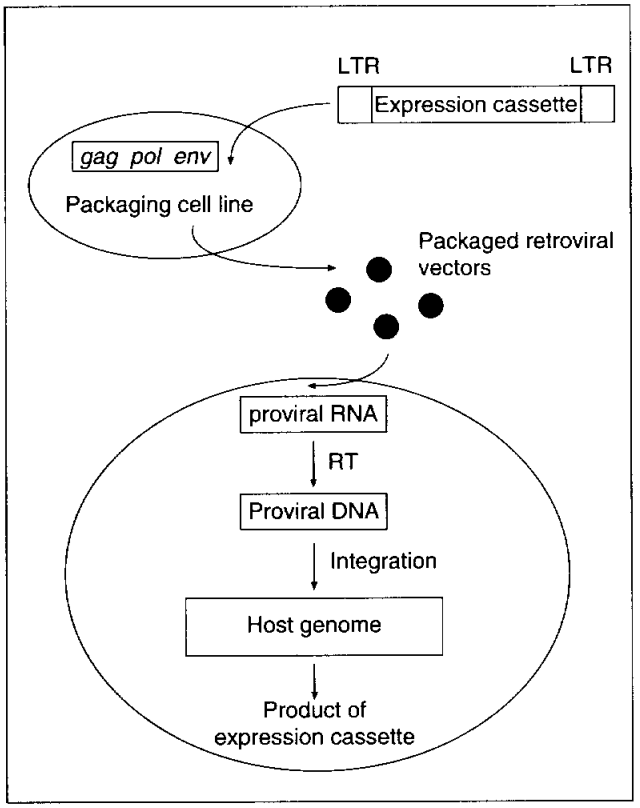

Fig 2. Retroviral vector structure, production, and gene transfer. Retroviruses are RNA viruses that replicate through a DNA intermediate step. The expression cassette is inserted between the retroviral long terminal repeats (LTR), and transfected into a packaging cell line containing the viral genomes *gag*, *pol*, and *env* that act in trans to provide the proteins necessary to package the virus. Replication-defective retroviral vectors containing the expression cassette enter the target cell via specific receptors. In the cytoplasm, the proviral RNA is reversely transcribed into DNA and integrated into the host genome. Normal cellular transcription then results in expression of the therapeutic gene.

Adenoviral vectors

Recombinant replication-deficient adenoviruses are more versatile vectors for gene transfer (Fig 3).[12] They are suitable both for in vitro and in vivo use because they can be produced in high titres (typically 10^{10} pfu/mL), transduce both dividing and non-dividing cells, and result in high expression of the therapeutic gene. Furthermore, the transferred gene remains epichromosomal, so that insertional mutagenesis is avoided (Table 1). Adenoviral vectors, however, elicit an antiviral cellular immunity as a result of the expression of viral proteins on the transduced cell. This host immunity, together with the epichromosomal location of the transferred gene, limit gene expression to several weeks. Repetitive administration may be required to maintain persistent gene expression but antibodies directed against adenoviral capsid proteins may limit the efficacy of this approach. Adenoviral vectors have been the main type of vector used in gene transfer in vivo.

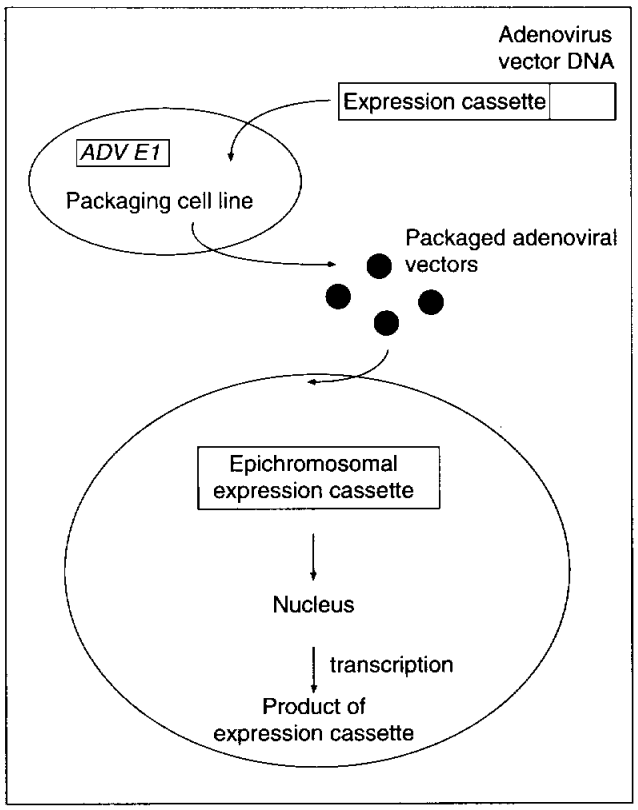

Fig 3. Adenoviral vector structure, production, and gene transfer. Adenoviruses are DNA viruses. The expression cassette is inserted into an adenoviral backbone containing a deletion of the E1 region that is critical for replication. The expression cassette is transfected into a packaging cell line that contains the adenoviral genome E1 that acts in trans to provide the protein necessary for viral replication. The replication-defective adenoviral vector enters the target cell through a specific receptor. The expression cassette then travels to the nucleus, remains epichromosomal and is transcribed, leading to expression of the therapeutic gene.

Plasmid-liposome complex

Plasmid-liposome complexes have a number of theoretical advantages (Fig 4).[13] These include the accommodation of genes of virtually unlimited sizes, the absence of risk of replication or recombination, and little or no host immunity is evoked. The disadvantage of this system, however, is that huge amounts are required to achieve meaningful gene transfer (Table 1). Because the transferred gene is epichromosomal, repeated administration is required; it is uncertain if this poses safety problems. Plasmid-liposome complexes are used mainly in gene transfer in vivo.

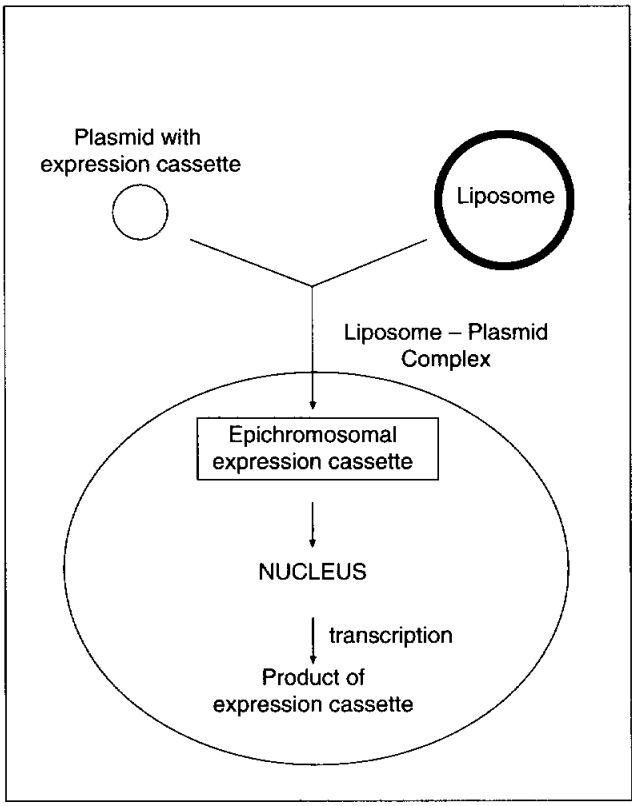

Fig 4. The structure of plasmid DNA liposomal complex and gene transfer. To increase stability and entry into cells, the plasmid DNA containing the expression cassette is fused with positively charged liposomes. The complex enters the target cell through fusion with the plasma membrane. The expression cassette then travels to the nucleus, remains epichromosomal and is transcribed, leading to expression of the therapeutic gene.

Table 1. Various vector methods of gene transfer

Vector	Application	Efficiency	Gene expression level/ duration of effect	In vivo safety
Retroviral vector	ex vivo gene delivery	good	high, permanent integration	insertional mutagenesis
	in vivo gene delivery	low, requires dividing cells	high, permanent integration, may be lost in vivo	insertional mutagenesis, recombination to RCV
Adenoviral vector	ex vivo gene delivery	good	high, short	expression of viral proteins
	in vivo gene delivery	good	high, short	expression of viral proteins, recombination to RCV
Plasmid DNA	ex vivo gene delivery	low	high, transient	-
	adenoviral coupled ex vivo gene delivery	high	high, transient	-
	in vivo gene delivery	low	high, transient	good
	liposome coupled in vivo delivery	low	high, short	good

RCV replication competent virus

Other vectors

Other viruses that have been used experimentally include adeno-associated virus,[14] vaccinia virus,[15] and herpes simplex virus.[16] The available data are not sufficient to assess their role in gene transfer in vivo.

Results of gene therapy studies

Although gene therapy has not yet resulted in the cure of any diseases, the experience gained from gene therapy trials has demonstrated that gene transfer is feasible, both ex vivo and in vivo. A variety of vectors, including retrovirus, adenovirus, and plasmid-liposome complexes have been shown to effectively transfer genes that result in relevant biological responses. The diseases that have been treated in gene therapy trials include both inherited and acquired genetic diseases.

Inherited genetic diseases

Early gene therapy trials have focused on the treatment of monogenic diseases, where mutation of a single gene causes the disease phenotype. The hypothesis of gene transfer in these conditions is that reconstitution of a normal gene to the appropriate sites should correct the disease. This approach has been tested in a number of clinical studies.

Severe combined immunodeficiency

Severe combined immunodeficiency (SCID) may be due to a number of genetic defects, one of which is mutation of the gene coding for the enzyme adenosine deaminase (ADA). This results in the toxic accumulation of adenosine and 2-deoxyadenosine in lymphocytes and consequently, in severely defective cellular and humoral responses. Retroviral-mediated ex vivo transfer of ADA cDNA into autologous T lymphocytes followed by subsequent re-infusion has resulted in partial reconstitution of the immune functions in recipients. Although the results are inconclusive because concomitant ADA infusion was also administered, these studies have verified the scientific principle of ex vivo gene transfer and serve as a paradigm for the treatment of other hereditary diseases.[17,18]

Familial hypercholesterolaemia

Familial hypercholesterolaemia is due to mutation of the gene coding for the low density lipoprotein (LDL)

Table 2. Clinical trials where immune-directed gene therapy strategies are used

Cytokines/Immune modulators	Means of gene transfer	Target tumours
IL-2	retroviral adenoviral lipofection	advanced cancers of the colon, brain, lung, ovary, prostate and kidney, melanoma
IL-4	retroviral	melanoma, advanced cancer
IL-7	retroviral electropolation	advanced cancers of the colon and kidney, lymphoma and melanoma
IL-12	retroviral lipofection	advanced cancer, melanoma
HLA-B7	lipofection	advanced cancer
TNF	retroviral	advanced cancer
GM-CSF	retroviral	melanoma, renal cell carcinoma
IFN-γ	retroviral	melanoma

IL interleukin; TNF tumour necrosis factor; GM-CSF granulocyte macrophage colony stimulating factor; IFN-γ interferon gamma

receptor, and results in high levels of serum cholesterol and LDL cholesterol that lead to fatal cardiovascular diseases. Retroviral ex vivo transfer of LDL receptor cDNA into hepatocytes followed by infusion via the portal vein has resulted in the reduction of LDL cholesterol levels and improved lipoprotein-related metabolic parameters.[19-22]

Cystic fibrosis

Cystic fibrosis (CF) is caused by mutations of the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR), which is a transmembrane protein that controls cAMP-regulated chloride transport. A deficiency of this protein leads to chronic airway infection and inflammation and progressive respiratory derangement. Early experiments have shown partial correction of the chloride transport defect when the CFTR is transferred to nasal epithelium in vivo.[23] Unfortunately, when a similar strategy employing adenoviral vectors was used in clinical trials, the results were disappointing—only molecular evidence of gene transfer at very high doses of therapeutic vectors was observed, but in the absence of functional improvement.[24] Another clinical trial using liposome-mediated gene transfer resulted in partial functional improvement, but results are far from conclusive.[25] Current research is focused on increasing the efficacy of gene transfer to airway-facing columnar epithelial cells, the critical target cells responsible for airway defence.[26]

Acquired genetic diseases

The most commonly acquired genetic diseases are tumours. Most genetic mutations involve point mutations, deletions, or gene fusions. These lead to the activation of oncogenes or inactivation of tumour suppressor genes, or formation of chimeric proteins, most of which are abnormal transcription factors. Several different strategies have been applied in the gene therapy of cancers.[27]

Suicide gene therapy

Suicide gene therapy involves the transfer of a therapeutic gene that confers pro-drug sensitivity only to transduced cells. The first example of a suicide gene is the gene encoding herpes simplex virus thymidine kinase (HSV-TK). This gene phosphorylates the anti-

viral agent ganciclovir (GCV), a derivative of acyclovir, into its monophosphate form.[28] Ganciclovir monophosphate is highly toxic to cells because when it is further phosphorylated it becomes a substrate for DNA synthesis that interferes with replication in dividing cells. Cells that do not express HSV-TK cannot phosphorylate GCV and are relatively insensitive to it (Fig 5). Furthermore, quiescent cells not engaged in DNA synthesis are also unaffected. Cancer cells genetically modified by HSV-TK have been shown to be effectively destroyed by GCV.[29] This approach has been termed "suicide" gene therapy (Fig 5).

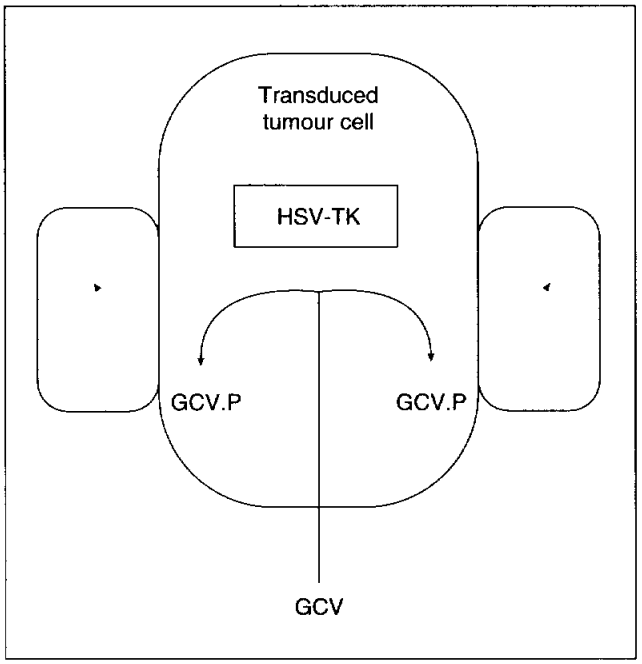

Fig 5. Suicide gene and the bystander effect. Tumour cells that are transduced with the suicide gene HSV-TK convert ganciclovir (GCV) into GCV monophosphate (GCV.P), which is highly toxic. Furthermore, GCV.P enters neighbouring non-transduced cells through tight junctions, resulting in death of these cells by a mechanism known as the bystander effect.

In murine models, when tumour cells transduced with HSV-TK in vitro were inoculated into the unmodified tumour in vivo, subsequent GCV treatment resulted in death of both transduced and non-transduced tumour cells, so that effective tumour regression was achieved.[30] Tumour regression was also observed when fibroblasts that had been engineered to produce retroviral vector carrying HSV-TK were inoculated directly into experimental brain tumour in vivo.[31] These experiments not only demonstrate the in vivo efficacy of the HSV-TK/GCV suicide gene approach, they also show that effective tumour regression does not require every tumour cell to be transduced. This phenomenon, in which non-transduced cells are killed by neighbouring transduced cells via toxic metabolites transported through gap junctions[32] or local cytokine production,[33] is known as the "bystander effect". It partially obviates the necessity of delivering the suicide gene to every tumour cell.

These earlier experiments involved the direct inoculation of TK transformed tumour cells or TK retroviral vector-producing cell lines into the tumours. To improve the efficacy of gene delivery, adenoviral vectors expressing HSV-TK (ADV/HSV-TK) were constructed and used for gene therapy experiments.[34] Using direct intratumoural inoculation of ADV/HSV-TK followed by GCV treatment, effective long term regression of experimental gliomas has been observed.[34] Successful demonstration of the in vivo efficacy of ADV/HSV-TK has resolved some of the major limitations associated with retroviral-producing cell lines for cancer treatment, so that the ADV/TK vector can be delivered effectively to tumours in different organs in vivo.

Immune targeting

Cancer cells are genetically mutated cells that may express abnormal proteins, or overexpress proteins that are normally cryptic or present in very small amounts. These proteins, known as tumour antigens, are potential targets for attack by the immune system.[35] The critical cells involved in the formation of an anti-tumour immunity are the CD4+ T helper cell, antigen presenting cell (APC), and CD8+ cytotoxic T cell (CTL). Tumour antigens are processed by APCs and presented in the groove of an MHC class II molecule to CD4+ T cells. This initiates a clonal expansion of the CD4+ T cells that leads to subsequent activation of tumour specific CD8+ CTL clones.[36] The process of antigen recognition is enhanced by the T cell co-stimulatory molecule CD28 in conjunction with its ligands B7-1 (CD 80) and B7-2 (CD86).[37] Activation of CD4+ T cells also leads to secretion of cytokines that up-regulate the tumour specific immunity. These cytokines include interleukin-2, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon-γ, and interleukin-12 (Fig 6).[36]

Most tumour cells, however, manage to evade the immune system by abrogating or minimising the anti-tumour immunity. Therefore, strategies designed to overcome these mechanisms may restore an otherwise silent

tumour-specific T cell response. Therefore, genetic manipulations to heighten immune awareness for recognition of unrecognised antigens on tumour cells, including up-regulation of MHC molecules and co-stimulatory molecules, and increased local cytokine production, may mobilise responsive T cells for destruction of these tumour cells.[38,39] Examples of these strategies include delivery of therapeutic vectors expressing interleukin-2,[40] GM-CSF,[41] B7-1,[42] and interleukin 12.[43] All of these strategies have resulted in the generation of anti-tumour immunity leading to the destruction of tumours in experimental animals. Most of these strategies have now been put into clinical trials (Table 2).

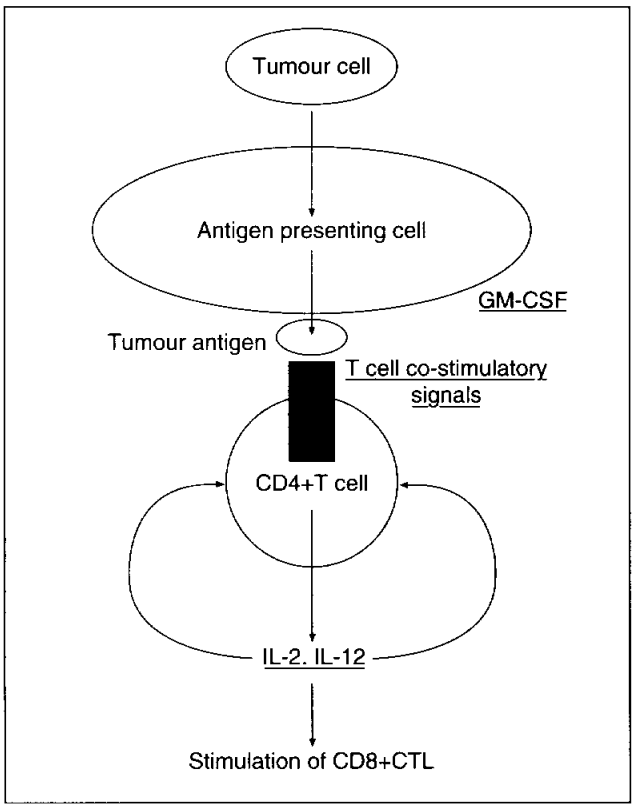

Fig 6. Formation of an anti-tumour immunity and how it can be up-regulated. Tumour cells are engulfed by antigen presenting cells and the tumour antigen is processed and presented on the cell surface. In conjunction with the T cell receptor and other T cell co-stimulatory molecules, CD4+ T helper cells are activated. These cells also secrete interleukin-2 (IL-2) and interleukin-12 (IL-12) that positively enhance T cell clonal expansion, as well as stimulate the expansion of tumour specific CD8+ cytotoxic T cells (CTL). Molecules that can enhance this process are underlined and they are potential target genes for immune-directed gene therapy.

Tumour-directed gene therapy

Studies of molecular genetics in cancers have revealed two main themes of somatic mutations—the deregulation of oncogenes due to mutations or gene fusion, and the inactivation of tumour suppressor genes.[44,45] Therefore, suppression of oncogenes or reconstitution of tumour suppressor genes represents potential strategies for reversing the mutated phenotype (Table 3). The suppression of oncogenes entails interference with the processing of genetic information by the use of antisense oligonucleotides or ribozymes.[46] Antisense oligonucleotides are designed to be complementary to a critical sequence in the transcript of a mutated gene, hence inhibiting its function. Ribozymes are RNA molecules that contain, in addition to an antisense sequence, an active catalytic site that cleaves a bound RNA molecule. Genes targeted in this way include bcl-2, IGF-1, and bcr/abl.[47] While these methods are effective ex vivo, for realistic utility in cancer gene therapy in vivo, high efficiency gene delivery systems of antisense/ribozymes are needed.

Reconstitution of a non-functional tumour suppressor gene is another way of abrogating the malignant phenotype. The most commonly mutated gene is p53.[48] In experimental models, reconstitution of the wide type p53 in cell lines with homozygous mutations of p53 inhibited tumourgenicity and restored chemosensitivity.[49] The mechanism of tumour suppression may be due to the restoration of apoptosis that is deficient in cells with homozygously mutated p53.

Other strategies

There are other gene therapy strategies that are currently being developed for cancer treatment, including the transfer of the multidrug resistance gene to haematopoietic stem cells for protection against high dose chemotherapy, and the use of replication competent viral vectors. These studies are preliminary and require further evaluation.

Hurdles of gene therapy

Early gene therapy trials focused on investigating the feasibility and toxicity of gene transfer. Most studies used retroviral vectors and the expression cassette was predominantly an antibiotic (neomycin) resistance marker gene. These studies successfully demonstrated that gene transfer to a variety of cells is feasible, including lymphocytes, haematopoietic stem cells, and neoplastic cells and that genetically marked cells can survive in vivo. More importantly, none of these studies showed undue toxicity, underscoring the safety of gene therapy in vivo. However, before gene therapy

Table 3. Tumour-directed gene therapy

Therapeutic gene	Means of gene transfer	Target tumours
p53 cDNA	adenovirus retrovirus	cancers of lung, colon, rectum, liver, head and neck
bcr/abl antisense	ex vivo purging	chronic myeloid leukaemia
c-myb antisense	ex vivo purging	chronic myeloid leukaemia
c-fos antisense c-myc antisense	retrovirus	breast cancer

can become a realistic treatment, several hurdles have to be overcome.

Translation of results from animal experiments to humans

The observations of efficacy of gene therapy in animals may not necessarily translate into clinical utility. Thus, although dramatic responses of tumour vaccines in tumour regression and protection against rechallenge had been observed in mice, the clinical results of this approach in humans were disappointing. In CF, although various gene therapy strategies worked very well in mice, in humans, the difficulties encountered in transducing bronchial epithelial cells with non-toxic doses of vectors have seriously limited clinical efficacy. Therefore, clinically relevant experimental models will have to be developed to test the theory and practice of gene therapy strategies. This is critical and will be one of the most important steps in a successful protocol.

Variations in the techniques of gene transfer and the observed results

Numerous approaches are used for gene transfer and each institute uses different techniques. This makes comparison of results between individual centres difficult. For these reasons, inconsistency in the efficiency of ADA cDNA and CFTR cDNA transfer has been observed. Therefore, further research should be directed towards the definition of what constitutes a clinically meaningful response and how this can be consistently evaluated.

Technical limits of current vectors

Currently available vectors have major limitations. Retroviruses are produced in low titres, require dividing cells to be effective, and may result in insertional mutagenesis. Adenoviruses result in transient gene expression and elicitation of host antiviral immunity. Plasmid-liposome complex carries the risk of endotoxin contamination. In addition, although toxicity may not be observed in animals, significant side effects might be seen in humans. An ideal vector that is capable of accepting expression cassettes of any size, is target cell-specific, not recognised by the host's immune system, stable, easily produced and purified in high concentrations, non-toxic, not associated with inflammation, and expressed in a regulated fashion for as long as is desired, is still not available.

Safety of gene transfer

The safety of gene therapy is an important issue that has to be addressed. Vector-induced inflammation, the possibility of recombination to form replication competent viruses, and the occurrence of insertional mutagenesis are the main problems. Concern that the therapeutic gene may enter the germline and hence the individual's gametes, and the potential risk of generating infectious vectors with powerful biological functions are more public issues.

Promises

Despite the presence of many conceptual and technical problems, gene therapy remains an important and innovative treatment modality. Evidence of its efficacy is compelling and ongoing research directed at improving efficiency, specificity, and safety has made steady progress. Although initially tested in inherited genetic diseases, gene therapy is becoming more and more important in the treatment of cancer, the most common form of genetic disease. Therefore, while gene therapy research in inherited diseases will still provide critical scientific insights, it is likely that more applications will be found in cancer treatment. Finally, whether modification of the germline human genome is possible and ethical will be contentions to be debated by society.

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