The genie in the test tube

– from gene to gene therapy: part II

In the previous edition, we discussed gene therapy in terms of potential vectors and their applications. Now, we discuss the potential clinical uses of these constructs. To date, the main applications of gene therapy have been in inborn errors of metabolism, cancer, cardiovascular and central nervous system degenerative disease and transplantation and graft versus host disease. However, it would seem that this list is set to grow as understanding of the fundamental causes of disease and of delivery mechanisms for gene therapy become better understood.

By Dr Jan Steiner

To date, the main areas of application of gene therapy have been in inborn errors of metabolism, cancer, cardiovascular and central nervous system degenerative diseases and transplantation and graft versus host disease. Intense efforts have also been made to utilise gene therapy in the control of HIV infection. However, it would seem likely that this list is set to grow as understanding of the fundamental causes of disease and of delivery mechanisms for gene therapy become better understood.

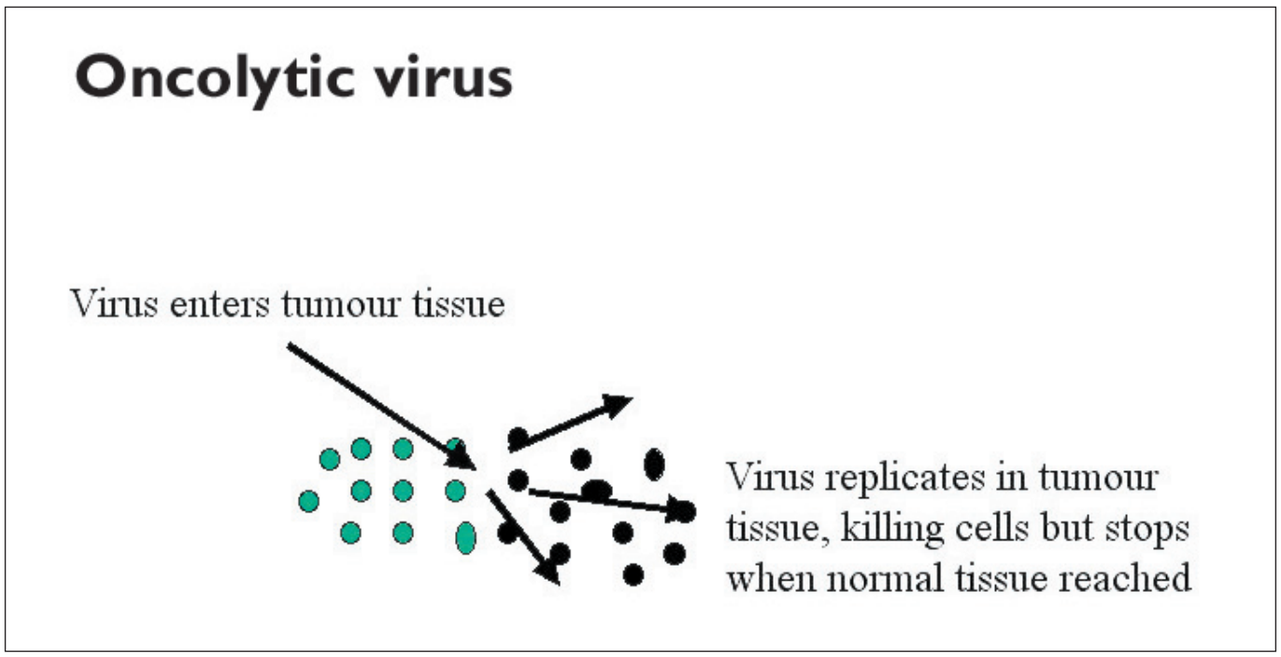
Inborn errors of metabolism

This term implies that the absence, relative deficiency or abnormality of one or more key molecules (typically enzymes or hormones) can produce a clinically important syndrome or phenotype. To date, the response to these abnormalities has been to supply the missing substance, as is the case with, for example, juvenile diabetes mellitus (insulin) or haemophilia (clotting factor VIII). In gene therapy, the issues have been to supply a therapy that produces an appropriately high level of gene delivery to the right tissues and lasting gene expression.

To take these two examples further, the abnormality in haemophilia resides in a single gene. The abnormality in juvenile diabetes, however, is believed to be multifactorial. This means that several genes, which may not lie close to each other in the genome, would seem to be involved.

In the first instance, it is relatively easy to supply the single gene for factor VIII. This is a therapeutic advance, since the current treatment for haemophilia with human factor VIII is risky (because of the potential for the transmission of HIV), short-lived and expensive (factor VIII, either human or recombinant, is naturally cleared from the blood stream over time). Not surprisingly, a number of companies are evaluating the possibility of introducing the gene for factor VIII as a cure for haemophilia. The situation is further improved by the observation that feedback controls are not apparently required for the secretion of factor VIII and that even a partial replacement (10% of normal in a severely deficient individual) is therapeutically worthwhile. So far, it is not known how long these treatments will last and how often they will need to be repeated.

By contrast, it has not yet been possible to introduce gene therapy remedies for juvenile diabetes mellitus. This relates to the perceived need to replace more than one gene, in the right tissue type and



under the right set of external controls. On the other hand, if the aetiology of diabetes mellitus turns out, as some authorities believe, to be an example of an autoimmune disorder (that is, the body's immune system, for various not yet understood reasons, turns against its own tissues), then a totally different therapeutic approach could be justified. This would involve manipulation of the immune system to prevent destruction of insulin producing cells.

There are other examples of the potential utility of replacing defective or absent molecules. These include the replacement of the gene coding for the enzyme, adenosine deaminase (ADA), in SCID, the gene for CFTR (cystic fibrosis transmembrane conductance regulatory channel that transports chloride ions) in cystic fibrosis and the gene for the hepatic LDL (low-density lipoprotein) receptor in familial hypercholesterolaemia.

These, like haemophilia, seem only to require partial replacement in order to obtain therapeutic gains. However, problems have arisen because it is still not possible to obtain long-term gene expression and because targeting the gene therapy product to the appropriate tissues is still difficult.

For example, in SCID, deficient lymphocytes are transfected ex vivo. In cystic fibrosis, the presence of the characteristic mucus in the lungs (caused by the lack of CFTR in lung tissues) makes topical delivery of the CFTR gene to the cells where it is needed, to overcome the deficiency, unreliable.

Cancer

This has turned out to be, so far, the area where the greatest numbers of gene therapy applications have been studied. This has been aided by the rapid progress in the understanding of the biology of tumour cells and the appearance of many new therapeutic targets. It seems inevitable that, over the next few years, the products of biotechnology and gene therapy will substantially change the treatment of cancer.

Several basic problems remain to be solved in the efficient delivery of genes to cancer cells. Even with local injection of the construct, gene transfer is generally patchy. The large number of cells involved and the presence of necrotic and/or fibrotic tissue within cancer masses further impedes the delivery of locally injected vectors. Delivery to distant metastases has not yet become a reality.

So far, the aim of conventional cancer therapy has been the eradication of all tumour cells when the disease is diagnosed early, either by surgery, radiotherapy and/or chemotherapy and the provision of palliation once the tumour has spread so that it can no longer be eradicated. The former has, in many instances been successful. The latter still leaves much to be desired in terms of therapeutic success and of associated quality of life.

The old, rather non-specific and highly toxic techniques of attempted cure or debulking of metastatic disease using cytotoxic agents will become modulat-

ed by the introduction of therapies that interfere with various pathways in the tumour cell cycle. These include oncogenes, cell signalling and growth factor pathways and induction of tumour suppressors and apoptosis (programmed cell death). In addition, the ability to introduce 'suicide genes' (eg thymidine kinase of cytochrome P-450 isoenzymes) should enable cytotoxic agents to work more effectively within tumour masses. In this instance, it may not be necessary to transfect all tumour cells with the suicide gene, since the activated cytotoxic agent may diffuse through the tumour mass, producing cell death as a bystander effect.

In addition, the ability to make the immune system aware of the presence of a tumour, which has previously been seen as self and not foreign, should enable the body's internal defences to deal more effectively with disseminated tumours. Fundamental knowledge of the induction of an immune response has shown us that tumour cells, by lacking the mechanism for manufacture and release of inflammatory cytokines, remain immunologically silent despite expression of immunologically 'foreign' surface markers. The reintroduction of the genes for these cytokines (specifically interleukin-2 [IL-2] and granulocyte macrophage colony stimulating factor [GM-CSF]) may aid the development of a normal immune response to abnormal cell surface markers.

In addition, the induction of immunity to 'normal' surface markers that are over expressed on tumour cells offers another opportunity in immunotherapy of tumours. Such normal epitopes include the carcino-embryonic antigen (CEA) marker in colorectal cancer, the CA125 marker in ovarian cancer, the MUC1 and her2/neu markers in breast cancer and various surface markers including the MAGE antigens in melanoma. To some extent, proof of principle for an immunotherapeutic approach has already been obtained with the use of monoclonal antibodies in the treatment of cancer. The recent approval of Herceptin®, a monoclonal antibody to her2/neu, for the treatment of advanced breast cancer illustrates the point.

In some of these instances, it is not yet clear whether sustained gene expression will be important or not. It has been argued that since, in cancer, it is only important to clear the cancer cells over a limited period of time, sustained expression may not be necessary. On the other hand, if we move to a model where the aim is to keep malignant cells under control rather than eliminating them entirely, then prolonged gene expression could become a vital asset.

Finally, the use of viruses with limited replication competence may provide a further therapeutic tool in the destruction of tumour masses and may overcome some of the previously mentioned difficulties encountered in delivery of vector to tumour cells.

These techniques are all in their infancy. So far there is no long-term information about efficacy or safety. Most importantly, the use of these various approaches in combination has not been extensively evaluated. Yet, on the basis of first principles, it would seem likely that multiple treatment regimens that attack various parts of the malignant cell cycle will be more successful than any of the potential treatments on their own. Further, the timing of such treatments, in relation to each other, may be of crucial importance.

While not exhaustive, the following examples characterise the advances that are being made in the development of gene therapies for cancer.

Head and neck squamous carcinomas

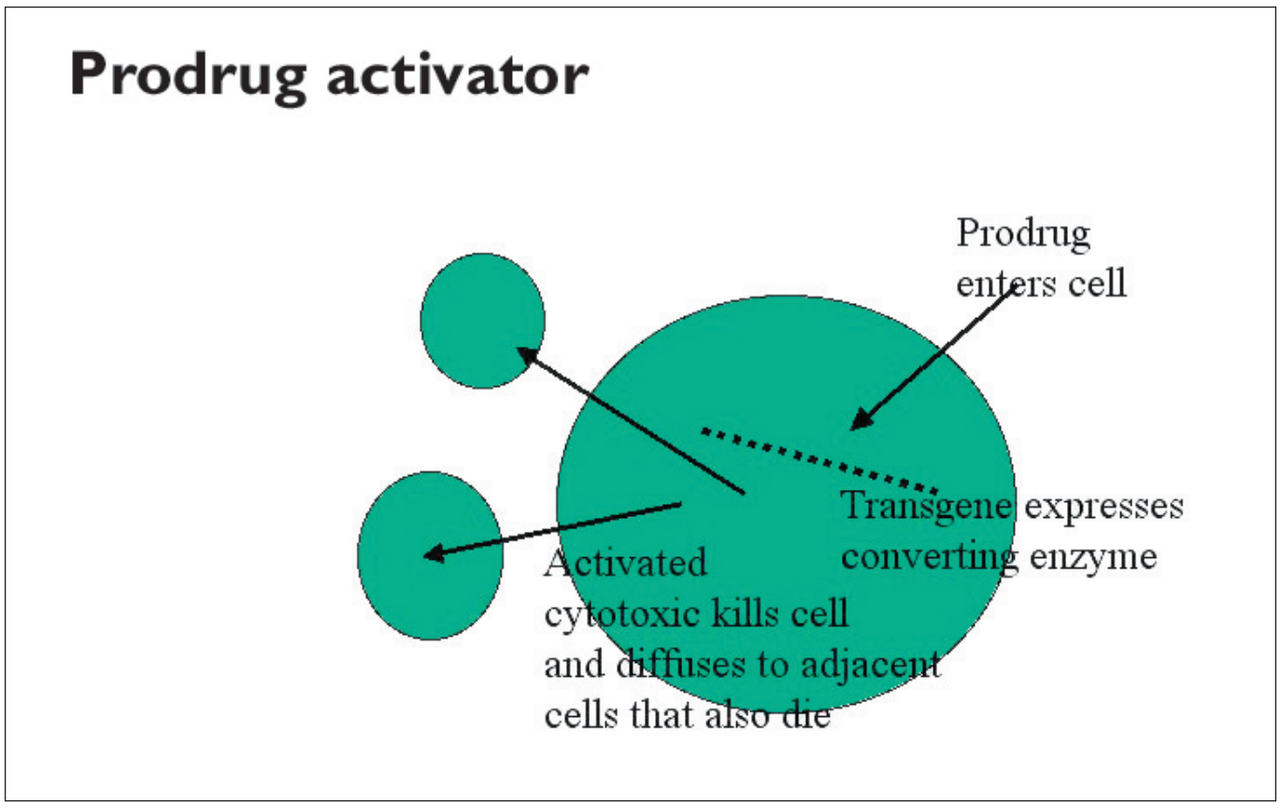
These are tumours that are commonly resistant to conventional chemotherapy. Surgical ablation may be difficult for deeply sited tumours in the nasopharynx and destructive in the case of tumours that are already large on presentation. These tumours therefore represent an area of high, unmet medical need.

A recombinant adenovirus carrying a herpes virus (HSV) thymidine kinase (TK) gene is in preclinical development to sensitise these tumours to ganciclovir. In this situation, the ability of the vector to transduce many tumour cells (not just those that are dividing, as would be the case with a retroviral vector) is perceived as an advantage. In addition, in this situation, the induction of an immune response is not seen as detrimental – indeed an inflammatory reaction may help to clear tumour cells.

Further refinements of this approach include the encapsulation of the adenovirus to provide for slow release into the tumour mass and the incorporation of the TK gene into an oncolytic (or partially replicating) virus in order to enhance efficacy.

An adenoviral vector engineered to deliver wild type p53 tumour suppressor gene is also in clinical trials (Ad5CMV-p53, Introgen Therapeutics) in a variety of indications, including head and neck cancer. As a single agent, it is capable of inducing tumour regression in some cases. Preclinical animal studies suggest that Ad5CMV-p53 may also have bystander effects and may act synergistically with chemotherapy (cisplatin) or radiotherapy.

ONYX-015 (Oxyx Pharmaceuticals) is an oncolytic adenovirus with a deletion of the E1B gene that allows the virus to replicate selectively in and to destroy cells lacking normal p53 gene function. An



early clinical study in patients relapsing after surgery and radiation assessed the safety and efficacy of ONYX-015 in combination with 5-fluoruracil/cis-platin. Although a high response rate was seen (62%), it is not possible to distinguish between the effects of the chemotherapy and the gene therapy at this stage. Further data are awaited from a randomised comparison of ONYX-015/chemotherapy and the same chemotherapy alone.

Other oncolytic viruses that are, or shortly will be, in the clinic include herpes viruses, Newcastle disease virus and reovirus. All have been shown to replicate selectively in cancer cells, although the mechanisms are not always fully understood. Reovirus is known to require the presence of an activated Ras signalling pathway.

Glioblastoma multiforme

A retroviral vector bearing the HSV TK gene is in clinical trials in patients being treated surgically for glioblastoma multiforme. This is the most aggressive of all primary brain tumours and the prognosis is still very poor. Typically, it is not possible to remove the entire tumour mass at surgery since it spreads into the surrounding normal tissues. In the past, radiotherapy and chemotherapy have been used to attempt to destroy these remaining malignant cells.

In these trials, the vector is introduced at surgery with the objective of transducing malignant cells that are dividing while sparing normal non-dividing neurones. The patients are then treated post-operatively with ganciclovir. This treatment should then destroy the malignant cell and the vector, thus providing an extra safety feature for this treatment.

Other approaches have involved the use of oncolytic herpes viruses that are injected directly into tumours. HSV specifically infects neural tissue and oncolytic viruses can be engineered to replicate only in tumour cells. So far, these vectors are in very preliminary clinical trials, the first being Neurovir's G207 vector. Issues of accurate and adequate delivery remain to be solved. There is also the (at least theoretical) safety concern, that these vectors could cause encephalitis or a non-specific inflammatory response that could prove detrimental. This is of particular importance in patients with glioblastoma, who are generally treated with high dose corticosteroids that limit immune responsiveness to viral infections. In addition, the interaction of these vectors with standard chemo- or radiotherapy remains to be explored.

Prostate cancer

In the USA, prostate cancer remains the most commonly diagnosed internal malignancy in men and the second leading cause of cancer death. Despite screening for prostate specific antigen (PSA), many cases are not diagnosed until late when they have become inoperable. Further, prostate surgery with its appreciable incidence of impotence, is not always ideal.

A variety of gene therapy approaches is now under evaluation, corresponding to the large number of tumourigenic mutations known to exist in this condition. These include replacement with the wild type p53 suppressor gene, restoration of glutathione-S-transferase and introduction of antisense c-myc and HSV TK. In addition, a number of immunotherapy strategies are being explored, including introduction of the cytokines, GM-CSF and IL-2 and induction of immunity to PSA since it is a tumour specific surface marker on prostate cancer cells. Retrovirus, adenovirus and vaccinia viruses are all being studied. Also, the oncolytic adenovirus ONYX-015 is in early clinical trials in prostate cancer

Pulmonary disorders

Cystic fibrosis

This is a disorder in which a single gene mutation alters the structure of the CFTR, a chloride ion pump that normally exchanges sodium and potassium across cell membranes. In the abnormal situation, various mucus-secreting tissues (in particular, the lungs and the small bowel) produce an extra viscous form of mucus that causes obstruction and, in the case of the lung, an added susceptibility to bacterial infection. This secondary infection has, in the past, been a prime cause of morbidity and mortality in children affected with this disease.

The presence of this viscid mucus has proved a barrier to the delivery of many therapeutic agents to the lung, including gene therapy vectors. These now include adenoviruses and non-viral delivery systems, both of which have been studied in phase I clinical trials. To date, problems with adenoviruses have been in terms of low levels of gene transfer and short-lived gene expression. The potential for immune responses, either producing neutralising antibodies that further limit the effectiveness of the vector or induce generalised 'flu-like symptoms or, more worryingly, inflammatory responses in the lung itself, cannot be ruled out and have not been overcome.

More recently, retroviral vectors have also been developed but these are still in preclinical development. Because of the ability of the retrovirus to incorporate into the genome of dividing cells, it may be possible to use these vectors to deliver genes to the developing lung, thus preventing the onset of structural damage that leads to chronic lung disease.

Chronic neurodegenerative disease

Parkinson's disease

In this disease, neurones containing dopamine, which regulate posture and muscle tone, degenerate. Sometimes the cause of this is cerebrovascular disease but, much more commonly, the precise cause is hereditary and still unknown. Whatever its origin, the disease is characterised by progressive tremor and immobility which eventually lead inexorably to chronic disability and death. Neurologists estimate that the disease does not become clinically apparent until about 90% of all dopaminergic neurones have been destroyed. Although treatment with L-Dopa, a precursor of dopamine has been standard for many years, it is ultimately unsatisfactory, since resistance and distressing side effects (on/off phenomena) become universal after long-term treatment.

Various vectors, including replication incompetent adenoviruses, adeno-associated viruses and herpes viruses loaded with genes for tyrosine hydroxylase and nerve growth factors are in preclinical development. In addition, research is being carried out with hybrid vectors such as a combination of AAV and HSV, in order to enhance neuronal uptake. As in the case of brain tumours, issues surrounding the accurate placement of the construct are of paramount importance, since the inadvertent destruction of the last functioning dopaminergic cells could be catastrophic.

Other important questions that have not yet been answered relate to the degree and duration of gene expression (what if persistent and untreatable on/off phenomena occur on the one hand and what if the therapeutic effect is transient on the other) and the potential for inflammatory or cytolytic responses in the brain.

An alternative approach, which has also been found to be effective in the delivery of replacement neurones, has been the use of foetal neural stem cells. The drawback of this technique is that a relatively large number of foetuses (6-12) is needed to obtain sufficient stem cells to produce a therapeutic effect. This renders this technique somewhat impractical. However, in the future, it may be possible to engineer more accessible cells to behave like neural stem cells, thus overcoming obvious problems of supply.

Cardiovascular disease

Hypercholesterolaemia has long been recognised as a risk factor for atherosclerosis and myocardial infarction. Standard treatments involve dietary control, weight loss and cholesterol lowering agents but these have limited utility and have to taken chronically.

Retroviral vectors encoding the gene for the low-density lipoprotein (LDL) receptor in the liver have been developed. This receptor plays a major role in the control of cholesterol levels in the circulation. However, the vector must be delivered by infusion into the portal vein. The duration and level of gene expression are therefore of great importance. In addition, the potential long term effects of the delivery of such a vector to tissues beyond the liver are as yet unknown.

Other possibilities include delivery of genes for apoproteins or lipoprotein lipase, enzymes that are also involved in the metabolism of cholesterol. These, however, are in a very early stage of research.

Further potential indications that are being studied in clinical trials include introduction of genes encoding for vascular endothelial growth factor (VEGF) for the local treatment of vascular insufficiency.

Clinical transplantation

Organ transplants with allogeneic (non self) tissues generate an immune response in the recipient that is mainly T-cell mediated. Although great strides have been made in the development of small molecule immunosuppressants, the outcome is not perfect in that rejection (30% at five years) and infections, cancer and ischaemic heart disease as a result of generalised immunosuppressive therapy may occur.

It is now understood that major histocompatibility complex (MHC) loci on antigen presenting cells of the immune system present foreign peptides on the cell surface and interact with T-cell receptors (TCR) on T-cells to initiate T-cell mediated immune responses. The mismatching of the MHC locus itself on the grafted organ can be expected to initiate immune responses and, therefore, graft rejection. In addition, a number of T-cell and cytokine mediated co-stimulatory signals are needed for graft rejection.

Various possibilities to interfere with the acute rejection process are being researched at present. All involve the delivery of a gene therapy product to the transplanted organ in order to induce local transplant tolerance. They include immunosuppressive cytokines such as viral IL-10 and cytotoxic T-lymphocyte antigen 4. Production by the liver of soluble donor-specific MHC complexes is also immunosuppressant, presumably because the complexes can bind with T-cells in the circulation or block the binding of T-cells in the graft.

In chronic graft rejection, smooth muscle cells proliferate in the vasculature of the transplanted organ and can lead to transplant atherosclerosis. Although the process is not fully understood, the involvement of the proto-oncogene c-myb, intercellular adhesion molecule 1 (ICAM-1) and loss of local endothelial nitric oxide synthase have been demonstrated in animal models. The delivery of the gene for nitric oxide synthase in an adenovirus vector and various antisense strategies to block the effects of ICAM-1 and c-myb are currently in research.

Further research efforts have targeted the genes for ribozymes (RNA molecules that act as enzymes). These include ribozymes that mediate graft versus host disease (GvHD), still a major problem following allogeneic bone marrow transplantation. These include molecules such as perforin and fas, both of which, when activated, initiate programmed cell death (apoptosis) and inflammatory cytokines such as interferon (or tumour necrosis factor).

HIV infection and AIDS

Over the last 10 years, small molecule treatments for HIV and AIDS have improved dramatically, with triple therapy regimens now decreasing mortality by up to two thirds, even in patients with low CD4+ cell counts. However, the dosing regimens are complex and expensive and need to be taken on an indefinite basis. The emergence of viral resistance continues to be a problem, especially among those individuals who do not comply regularly with treatment regimens. The search for a simpler treatment modality is therefore important.

Greater understanding of the HIV life cycle has identified various targets for therapy. Briefly, it is now known that the virus enters cells by specific protein-protein interactions between the virion surface (the gp120 surface subunit and the gp41 transmembrane subunit) and cell membrane surface receptors. The first of these to be identified was CD4+ but, subsequently, macrophages have been found to exhibit another receptor called CCR5 (chemokine receptor 5) and T-cells have been found to have an important receptor, CXCR4. It has now become apparent that the CCR5 receptor is crucial in allowing macrophages to be susceptible to HIV infections, since inherently resistant individuals are homozygous for a variant receptor, CCR5-Delta 32. In addition, it has become apparent that HIV-1 normally infects macrophages first. As it mutates during its life

cycle, it becomes able to infect T-cells via the CD4 and CXCR4 receptors, causing T-cell depletion and the emergence of AIDS defining illnesses.

The viral RNA genome converts from RNA to DNA by the formation of reverse transcriptase (RT) and integrates randomly into the host cell genome as a provirus flanked by long terminal repeats (LTR). Once there, it is able to transcribe for a number of transcription factors and viral gene products including trans-activator of transcription (tat), regulator of viral proteins (rev), group antigen (Gag), virion infectivity factor (vif) and the Nef protein, which docks with MHC1 proteins. Transcription is initiated at the 5 LTR (the viral promoter) and terminated at the 3 LTR. Tat binds with a protein structure called the TAR-element that is believed to promote the overall rate of viral transcription. Rev binds with an RNA secondary structure called the RRE-element, present within the envelope, to move viral replication from its early double spliced to late stage single spliced mRNA. These two sites have provided an early focus for the inhibition of HIV replication.

Initial gene therapy strategies included the ex vivo transduction of lymphocytes with env (envelope) and rev genes in a non replicating retroviral vector (HIV-IT(V); Chiron/Viagene), inhibitors of the rev gene and the gene for a ribozyme that cleaves HIV-1 RNA (Targeted Genetics Corp). Such strategies are cumbersome and are being replaced with in vivo techniques. More recently, however, Systemix, Inc has announced a clinical trial of transduction of human stem cells with vector encoding the rev M10 gene, which is designed to inhibit viral replication.

Examples of in vivo delivery include the introduction of antisense molecules such as Hybridon's GEM-91 Gag antisense gene, which ultimately turned out to be ineffective. More recently, gene therapies against other parts of the replicative cycle are under evaluation. So far, a tat-nuclease has been investigated, which should bind to and cleave all HIV RNA transcripts. A recent press release from the Children's Hospital of Philadelphia reported that an antitat antisense transgene in a murine retroviral vector was used to transduce peripheral blood mononuclear cells from infected patients. This led to inhibition of viral activation and replication ex vivo and prolongation of CD4 T-cell survival in these patients.

Other initiatives have involved the use of ribozymes to various viral targets and the use of sense 'decoys', nucleic acids that bind to proteins such as TAR is in research. While some promising in vitro data have been obtained for TAR decoys, these are still far from the clinic.

In addition, research is under way into gene therapy approaches to HIV vaccination. These include a canary pox vector engineered with multiple HIV gene products (sponsored by the National Institutes of Health). Nucleotide sequencing of env and gag genes has shown that these viruses are heterogeneous and has led to classification of the virus into a major or M group and a genetically distinct O group. Among the M group, there are at least nine genetically distinct subtypes termed clades.

Clade B appears to be more common in North America and Europe whereas clades A and D are more common in sub-Saharan Africa and clade E in South East Asia. These differences which lead to antigenic differences between the clades are key in determining whether a single vaccine can be used universally or whether a polyvalent vaccine will be necessary. Clearly, there is a long way to go. The immune response needed to prevent or abort infection is not understood and animal models are not predictive. So far, no HIV preventive vaccine has been successful.

Conclusion

This short review is intended to give an overview of some of the major thrusts in gene therapy today. Inevitably, it has not covered everything, since the research effort is immense and many interesting experiments in vitro do not ultimately have activity in vivo. While critics have claimed that, so far, there has been very little to show for the amount of work done in terms of successful drug candidates, it seems to be only a matter of time until successful medicines emerge.

As the numbers of molecular targets increase, so the numbers of potential therapies enlarge. Major issues still involve accurate delivery to target organs, duration of gene expression and safety concerns. Further hurdles will be the development of these therapies in conjunction with established small molecule and biopharmaceutical medications and with new entities now in development. The final comment has to be 'watch this space'. DDW

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