The genie in the test tube
– from gene to gene therapy

For thousands of years, medicine was a descriptive art, with treatments for disease arising from astute observation and trial and error. The rise of modern forms of biological sciences, especially the developments in molecular biology over the last 30 years, have allowed much more fundamental understanding of how cells, both normal and abnormal, work and how, also, normal cells become dysfunctional. In the first of a two-part article we discuss some of the issues that are specific to the development of the products of gene therapy.

The appreciation that genetic factors play important roles in causing many diseases has led research workers to explore the possibility that missing or defective genes could be replaced. This culminated in the first gene therapy treatment, which was for a rare but lethal disease called severe combined immune deficiency (SCID) or 'bubble boy' disease. This disease lent itself to early gene therapy procedures in being a single gene defect and in being a disease of blood cells, which are easy to obtain and to manipulate outside the body.

Although the early perception was that the techniques of introducing new genetic material into cells would largely be useful for the treatment of rare inborn errors of metabolism, (genetic replacement therapy), more basic knowledge of cellular processes has infinitely broadened that view. In many diseases, and most particularly, just now, in cancer, research has revealed the abnormalities and the causes of those abnormalities at a biochemical level. This has enabled imaginative approaches to a wide variety of pathologies. These include simple gene replacements or the introduction of therapeutic genes (as in the introduction of cytochrome P450 or thymidine kinase into cancer cells), manipulation of the body's own resources to treat disease (as in various forms of immunotherapy) and interference with the genetic mechanisms in abnormal cells.

If the introduced gene is the therapeutic moiety, then it needs a delivery mechanism to introduce it into cells and to make it work. There are several aspects to this. The first is to introduce the genes into appropriate cells. The second is to ensure that the gene finds expression at the right level and for the right period of time. The third is to ensure that genes do not distribute to tissues where their presence would be undesirable, especially in cells that can be passed on to future generations. In order to achieve this, vectors or delivery mechanisms have been developed.

The rest of this article will deal with the vectors that are currently being explored, the potential uses of such vector/gene complexes and the clinical areas that are opening up for research and development.

Vectors

The earliest studies bombarded cells ex vivo with naked DNA. This approach had several drawbacks, including a lack of sustained gene expression in the target tissues and a limitation in the clinical application of such technology. However, the application of molecular biology to virology led to an increased understanding of the physiology of viral genomes and how they could be harnessed to provide the

By Dr Jan Steiner

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ideal vector. Viruses have natural advantages in terms of tissue specificity, penetration of and trafficking within cells, delivery of single or multiple gene payloads, potential for incorporation into the host cell genome, or not, and a differentiation between sustained or transient gene expression, depending on the circumstances.

The earliest viruses to be studied as vectors were retroviruses and adenoviruses. Both are normal human pathogens, the former including the HIV virus and the latter causing upper respiratory tract infections or colds. Subsequently many other viruses, both human and non-human pathogens, have been explored for their properties as vectors. To date, there is no 'universal vector'. Rather, the appropriate vector must be chosen on the basis of its benefits and risks.

For instance, concerns have been raised about the potential for viruses to replicate and to cause host tissue damage. This anxiety also applies to those viruses that can incorporate into the genome of the host cell to cause abnormalities. This may occur by virtue of their potential for the switching on of oncogenes and the recombination with wild viruses, which may be present in the host, to cause overwhelming infections.

Progressive steps to disable the replicative powers of viruses have been employed to address these issues. Indeed, most vectors these days are so replication incompetent that they must be grown up in cell media that have been engineered to provide the missing factors that allow the virus to multiply to titres that can be commercially viable. The removal of the genes that encode for the replicative processes has a second advantage in that the space created can be filled with one or more therapeutic transgenes.

Steps taken to render viruses incapable of replication include removal of the gag, pol and env gene regions in the MMLV (see below). The gag region encodes the capsid proteins, the pol region encodes for reverse transcriptase and the env region encodes for receptor recognition and envelope anchoring. Once these regions are removed, the vector can integrate itself into the host genome but cannot reproduce spontaneously. Adenovirus vectors are depleted of their E1 region, which is essential for reproduction and may also be deprived of the E3 and E4 regions in order to allow insertion of extra transgenes. Herpes viruses are made non-neurovirulent by removal of the ICP34.5 gene.

By contrast, it has been realised that, in certain cases, limited or conditional replication competence is an advantage. In these cases, the virus is provided with the ability to replicate, so long as host cells can provide it with certain specific growth factors. These factors may be, for example, limited to tumour cells. The idea is that the virus then replicates in and destroys tumour cells but is unable to grow in normal tissues. In this way, the vector, in itself, becomes a therapeutic agent. An example of this is the ONYX-015 adenovirus that is currently in clinical trials in a variety of solid tumours. However, this is not the end of the story, for the vector can also, of course, introduce genes for other proteins that could assist with tumour cell destruction.

So far, these techniques have primarily been applied to cancer. However, potentially, various other pathological cell proliferative states, including coronary artery restenosis, could benefit from such an approach.

A further concern, highlighted by the recent fatal case of Jesse Gelsinger, in the USA, involves the potential for overwhelming and lethal immune responses to vectors that are commonly present in the population. This applies to the adenovirus vector with which Gelsinger was injected, since adenovirus antibodies are frequently found in healthy humans. Similar considerations may apply to other common human pathogens such as the herpes virus and require careful evaluation. As a further consideration, the presence of pre-existing neutralising antibodies to a vector may substantially limit its survival in the body and, thus, its therapeutic utility.

The list of viruses that are being pressed into service continues to grow. Retroviruses, adenoviruses and adeno-associated viruses (AAV) have been with us for some time. More recently, herpes viruses, pox viruses (including smallpox and canary pox), Newcastle virus (a disease of chickens) and reovirus have also emerged as potential contenders for certain indications.

The main concerns to consider in any viral vector are as follows:

1 Which tissues will it target?
2 Will it induce an immune response?
3 Will it incorporate into the genome? If so, will that incorporation be random and cause problems?
4 Can it incorporate into dividing or non-dividing cells?
5 At what level and for how long will gene expression be maintained?
6 What is the size of the payload?
7 What is its potential for viral recombination and oncogene formation?
8 What special properties does it have that make it applicable to certain disease states?
9 Can it be produced in commercial quantities and in a cost-effective way?

The most commonly used viruses currently in clinical trials are retroviruses. These are RNA viruses, including the Moloney murine leukaemia virus (MMLV), the C family of human endogenous retroviruses (HERV-C) and lentiviruses such as human immunodeficiency types 1 and 2 (HIV-1 and HIV-2). The diameter of the virion ranges from 80nm to 130nm, the genomes consist of two positive-sense single-stranded RNA molecules, sized 3.5kb to 10kb. The genomes are encased in a capsid along with integrase and reverse transcriptase (RT) enzymes. Retroviruses only transduce cells that are actively undergoing mitosis and are therefore well suited for pluripotent or malignant cell gene transfer protocols.

Lentiviruses have the advantage over MMLV in that they can transduce non-dividing cells as well as those that are actively dividing. They integrate stably into the host genome and have an inherent affinity for CD4+ T-cells, macrophages and human stem cells. This may give these vectors great potential in the treatment of AIDS and HIV infection. In addition, modifications of the lentiviral genome can be used to increase the tropism of the vector so that the vector now has potential for the gene therapy of cystic fibrosis (airway epithelial cells) and Parkinson's disease (fully differentiated neurones).

Adenoviruses are non-enveloped, icosahedral, double-stranded DNA viruses with a capsid diameter of 70-100nm and a genome of 36kb. They are not incorporated into the genome of the target cell but remain as extrachromosomal entities in the nucleus of the host cell. This is potentially an advantage since there is no chance of activation of oncogenes or interference with tumour suppressor genes.

They are normal human pathogens (47 serotypes have so far been identified) and infections produce symptoms ranging from colds to acute febrile pharyngitis (sore throats). The disadvantage that this confers is the likelihood that recipients of the vector will be pre-primed and may mount a vigorous memory immune response. Two serotypes (Ad2 and Ad5) have so far been the most used for gene therapy studies.

The genome is divided into two main regions expressing four early (E1, E2, E3, E4) and five late (L1, L2, L3, L4, L5) genes. The early genes initiate viral replication and shut down host protein synthesis. The late genes code for the polypeptides that are required for virion assembly.

The transduction efficiency is high but the period of expression of the transgene is relatively short. This is partly due to immune destruction of the construct and also because, having not been incorporated into the host genome, the construct will not divide as the host cells divide and will be left in just one of two daughter cells. Thus, adenoviral vectors are primarily indicated when one-off, short-term gene expression is required.

Adeno-associated virus (AAV) vectors offer a wide host cell range and potentially high induction efficiency. Unlike the adenovirus, AAV is a parvovirus and normally does not cause cell damage and integrates stably into the host cell genome at specific sites in certain circumstances, offering the benefit of long-term gene expression.

Herpes simplex viruses (HSV) specifically target neuronal tissue and have been developed for various neurological indications including Parkinson's disease and primary brain tumours. In addition, HSV can grow efficiently in many types of human malignancies including cultures of colorectal, prostate, squamous cell and non small cell lung cancers. HSV are DNA viruses that maintain themselves as extrachromosomal DNA elements in the nucleus of host cells, allowing for long-term gene expression. The wild type human pathogen normally establishes itself chronically within neural tissue, only reactivating itself in response to certain external stimuli. This property offers the possibility of introducing long-term gene expression in situ. The innate ability of herpes viruses to traffic along neurones also opens up the possibility of delivering genes to previously inaccessible sites in the central nervous system such as the dorsal route ganglion, in the treatment of pain, and the substantia nigra, in Parkinson's disease.

HSV vectors have a large genome of 153kb allowing them to accept large or multiple gene inserts. Like adenoviruses, deletion of immediate early genes that code for viral replication processes renders these viruses replication incompetent. A further advantage is that effective anti-herpetic medicines exist, which, in an emergency, could be used to terminate or abrogate unwanted activity.

Vaccinia (pox) virus has in the past been used as a live vaccine to eliminate small pox. As such, it has been administered to large populations and is known to be safe. Vaccinia virus does not integrate into host genomes and can incorporate large and multiple transgenes. Although immunogenic, these vectors are safe for single use, since small pox was eradicated 30 years ago and few members of the population still have pre-existing antibodies to the virus. On the other hand, the immunogenicity of the virus makes it useful in the development of tumour vaccines since genes for tumour antigens and inflammatory cytokines can be cloned into these vectors and expressed on the surface of the cells (either tumour or normal) into which they are injected.

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Table 1
Properties of current viral vectors

VIRUS	TISSUE SPECIFICITY	IMMUNITY ALREADY PRESENT	INCORPORATE INTO GENOME	DIVIDING OR NON-DIVIDING ELLS	EXPRESSION CHARACTERISTICS	SIZE OF PAYLOAD	SPECIAL PROPERTIES
Retrovirus	No	No	Yes	Dividing	Long lived	Small	–
Adenovirus	No	Yes	No	Non-dividing	High, short lived	Small	–
AAV	No	No	Yes	Non-dividing	Long lived	Small	–
Lentivirus	No	No	Yes	Both	Adenovirus	?	–
Herpesvirus	Yes	Yes	No	Non-dividing	High	Large	–
Poxvirus	No	Yes	No	Non-dividing	High, short lived	–	–
Newcastle virus	No	No	?	Non-dividing	–	Not important	Conditionally replicative
Reovirus	No	No	?	Non-dividing	–	Not important	Conditionally replicative

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Table 1 illustrates some of the properties of current viral vectors.

Delivery of viral vectors can be either ex vivo or in vivo. To date, most ex vivo techniques have involved the harvesting of blood or tumour cells, transfecting them with a transgene carrying vector and reinjecting them into the donor. In some instances, tumour cells have been fused with other cells, such as antigen presenting cells of the immune system, in order to create tumour vaccines.

In vivo delivery is more challenging in that, to date, problems of tissue specific systemic delivery have not been solved. In the scenario where such specificity is not important, as, for example in haemophilia, this is not an issue. However, for specific delivery, researchers have so far resorted to direct injection of gene therapy into the target tissue. Some of these techniques may be extremely invasive as in the case of the delivery of vectors to the liver via portal vein injection or to the pancreas via endoscopy. Again, this may not be a major issue if repeat treatments are not required. However, until now, targeted systemic delivery remains an unresolved problem.

In addition, various non-viral vectors are being evaluated. Broadly speaking, these avoid the concerns about viral recombination. However, their lack of specificity and their inability, so far, to deliver prolonged gene expression, have proved to be drawbacks. The argument has been made that the lack of specificity prevents the incorporation of the gene into the right cell where it will be under the right set of intracellular feedback controls. However, there are circumstances, such as haemophilia, mentioned previously, where a reasonable level of sustained gene expression, regardless of its tissue of origin, could be very welcome. On the other hand, in conditions, such as diabetes mellitus, in which diurnal rhythms or rapid responsiveness to external stimuli are necessary, incorporation into the right tissue with the right set of controls becomes critically important.

Current non-viral technologies under evaluation include local injection of naked DNA or DNA mounted on to a matrix such as gold beads, liposomes and DNA complexed to receptor ligands in an attempt to make gene delivery more tissue specific. To date, the most promising of these would seem to be a simple intramuscular injection. Genes can be injected that either express therapeutic proteins such as coagulation factor VIII or induce the cell to express various antigens on its surface. There are various examples of the latter approach being used to induce the expression of tumour markers that are then seen by the immune system as foreign. This can then form the basis of a therapeutic cancer vaccine.

In addition, the development of nanoparticles that contain vector constructs inside a protective shell, act as delivery mechanisms and protect them from immune recognition and destruction, when they are introduced into the circulation, is under way.

Finally, the use of transfected whole cell transplants is under evaluation. This could apply, for example, to engineered pancreatic cells that could be used to produce insulin in insulin-dependent diabetics. One of the major issues so far has been the need to overcome immune tissue rejection phenomena, similar to graft rejection. Such cells have been administered enclosed in protective coverings that remove them from immune surveillance. The technology is still in its infancy, however.

Potential uses

The original vision was to be able to replace a single defective inherited gene. So far, potentially promising results have been obtained in adenosine deaminase (ADA) deficiency, the cause of SCID disease and in haemophilia. In both of these disorders, the problem is fairly straightforward. In SCID, the defective gene can be introduced into affected blood cells ex vivo and reinjected into the body. In haemophilia, the gene product, coagulation factor VIII, can be effective even if not produced in its natural site, the liver.

On the other hand, many single gene deficiencies are not so easily repaired because the gene product must interact with other cellular elements in the right way or be subject to cellular feedback in order to be effective. For example, although the defect in sickle cell anaemia is a single gene defect in one strand of haemoglobin, this strand must combine correctly with a second strand in order to make an effective haemoglobin molecule. This then demands that the gene be introduced not only into red cells, but in the right part of the red cell's genome. This has not so far been achieved.

A more modern vision has been the replacement of genes that have mutated into an inactive form during cell division or been lost during some degenerative disease process. A classic example of mutagenicity is the p53 tumour suppressor gene in malignant cells. Many tumours express mutant forms of this protein. One avenue under exploration at present is the replacement of the mutant gene with the wild type, introduced by a viral vector. An added refinement would be to insert this gene into the genome of a conditionally replicative virus, so that the wild type p53 is spread throughout the tumour as the vector replicates throughout tumour cells. Doubtless, as the biology of malignant cells becomes more and more understood, many similar targets

will be identified. It is possible to imagine that several replacement genes could be introduced into a capacious vector and returned to a tumour.

The implication of this approach is that much more refined diagnostic techniques may be needed to pinpoint the defects in individual tumours. A second unresolved issue for any one case is whether all tumour deposits express the same genotype (the answer is frequently 'no') and what to do when different metastatic deposits are phenotypically different.

By contrast, the replacement of enzymes lost in degenerative diseases may be ostensibly more straightforward. For example, there is much interest in the replacement of the enzyme tyrosine hydroxylase, which is lost in Parkinson's disease. However, it may not be enough to replace enzyme when cell death is extensive. This has led researchers to seek to encourage nerve regrowth by introducing nerve growth factor genes into the part of the brain that is affected by Parkinson's disease. A similar experiment is being tried in Alzheimer's disease. The precise placement of the vector constructs into the brain is a key issue, as are concerns about the destructive potential of any inflammatory or cytolytic response to the viral vector. However, these difficulties may pale into insignificance when compared to locations that are even more inaccessible.

Another idea that is being widely developed is the introduction of the so-called 'suicide gene'. This is a gene that is able to activate a pro-drug within a cell. The active drug product is then available within the affected cell at relatively high concentration. This enhances local efficacy while avoiding generalised toxic effects. Typically, this approach has been applied to cancer. Both the gene for cytochrome P-450 2B6 that activates cyclophosphamide or iphosphamide and the gene for thymidine kinase that activates ganciclovir are under clinical evaluation in solid tumours. One great advantage of these therapeutic models is that they are pharmacologically silent until the enzyme substrate is introduced.

A further variant on this theme is to introduce a gene, which remains silent until a promoter (a substance that activates gene expression), such as rapamycin, is provided externally. Likewise, it should be possible to introduce external gene suppressors, in order to turn off therapeutic genes when they have outlived their usefulness. Clearly, the main issue here is to obtain sufficient specificity such that normal tissues are not also affected.

Another refinement of the use of gene technology is the introduction of immune modulation. This has been achieved in a number of ways. In its

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simplest incarnation, naked DNA expressing a tumour surface antigen or a vector containing the gene for the antigen has been introduced into a tissue such as muscle. The muscle cells then express the antigen. This cell surface antigen is detected by the immune system, which then mounts a response. The objective, of course, is that this response should be against the tumour rather than against other normal host cells, since the latter could induce autoimmune disease.

In order to make this response more specific, the antigen can be introduced directly into the special cells of the immune system, the dendritic cells that normally pick up and present foreign cellular antigens to the immune system. This can be done either by bombarding these cells with the antigen, by fusing them with tumour cells that contain the antigen or by transfecting them with a vector containing the gene for the antigen in question. In addition, it should be possible to combine into such vectors immunostimulatory genes such as genes for IL-2 or GM-CSF that will enhance the immune response to the presented antigen. All of these are highly experimental techniques at present, with large issues to be resolved about the manufacture, quality control and efficacy of the approach. However, these examples give a good feeling for the potential of the technique, once practical issues have been resolved.
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Jan Steiner PhD is a clinical pharmacologist who has worked in the pharmaceutical industry since 1981. In 1992 she founded Oxford Therapeutics Consulting Ltd, a company dedicated to assisting small biopharmaceutical firms with the strategic planning and implementation of product development plans. The company's experience covers a broad range of products from chemical entities, through biopharmaceuticals to the products of gene therapy.