HUMAN GENE THERAPY

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Although gene therapy as a treatment for disease holds great promise, progress in developing effective clinical protocols has been slow. The problem lies in the development of safe and efficient gene-delivery systems. This review will evaluate the problems and the potential solutions in this new field of medicine.

The first approved clinical protocol for somatic gene therapy started trials in September 1990(Ref 1). Since then, in just 7 1/2 years, more than 300 clinical protocols have been approved worldwide and over 3,000 patients have carried genetically engineered cells in their body. The conclusions from these trials are that gene therapy has the potential for treating a broad array of human diseases and that the procedure appears to carry a very low risk of adverse reactions; the efficiency of gene transfer and expression in human patients is, however, still disappointingly low. Except for anecdotal reports of individual patients being helped, there is still no conclusive evidence that a gene-therapy protocol has been successful in the treatment of a human disease. Why not?

In this review I will examine the 'why not?' by evaluating the promise and the problems of gene therapy. There are various categories of somatic cell gene therapy, distinguished by the mode of delivery of the gene to the affected tissue (see Box 1). The challenge is to develop gene therapy as an efficient and safe drug-delivery system. This goal is more difficult to achieve than many investigators had predicted 5 years ago. The human body has spent many thousands of years learning to protect itself from the onslaught of environmental hazards, including the incorporation of foreign DNA into its genome. Viruses, however, have been partially successful in overcoming these barriers and being able to insert their genetic material into human cells. Hence the initial efforts at gene therapy have been directed towards engineering viruses so that they could be used as vectors to carry therapeutic genes into patients. A number of reviews on aspects of gene therapy have been published recently (2-10); this review will consider the categories of the various virus vectors in turn.

Box 1 The three categories of somatic cell gene therapy

- The first is *ex vivo*, where cells are removed from the body, incubated with a vector, and the gene-engineered cells are returned to the body This procedure is usually done with blood cells because they are the easiest to remove and return.

- The second is *in situ*, where the vector is placed directly into the affected tissues. Examples are the infusion of adenoviral vectors into the trachea and bronchi of patients with cystic fibrosis, the injection of a tumour mass with a vector carrying the gene for a cytokine or a toxin, or the injection of a vector carrying a dystrophin gene directly into the muscle of a patient with muscular dystrophy,

- The third is *in vivo*, where a vector could be injected directly into the bloodstream. There are no clinical examples of this third category as yet, but if gene therapy is to fulfil its promise as a therapeutic option, *in vivo* injectable vectors must be developed.

Vectors based on RNA viruses

Retroviruses were initially chosen as the most promising gene-transfer vehicles. Currently, about 60% of all approved clinical protocols utilize retroviral vectors. These RNA viruses can carry out efficient gene transfer into many cell types and can stably integrate into the host cell genome (Fig. 1), thereby providing the possibility of long-term expression. They have minimal risk because retroviruses have evolved into relatively non-pathogenic parasites (although there are exceptions, such as the human immunodeficiency viruses (HIV) and human T-cell lymphotropic viruses (HTLV). In particular, murine leukemia virus (MULV) has traditionally been used as the vector of choice for clinical gene-therapy protocols, and a variety of packaging systems to enclose the vector genome within viral particles have been developed. The vectors themselves have all of the viral genes removed, are fully replication-defective and can accept up to about 8 kilobases (kb) of exogenous DNA.

The problems that investigators face in developing retroviral vectors that are effective in treating disease are of four main types: obtaining efficient delivery, transducing non-dividing cells, sustaining long-term gene expression, and developing a cost-effective way to manufacture the vector.

Obtaining efficient delivery. Clinical protocols with retroviral vectors primarily use the *ex vivo* approach. Currently, the cells that are transduced by retroviral vectors are those that possess a high level of the natural MuLV (amphotropic) receptor and are actively dividing at the time of exposure to the vector. Most human cells that can be grown *in vitro* can be transduced, although a few cell types cannot. An important target cell is the primitive haematopoietic stem cell (HSC) because gene transfer into these cells would result in gene-engineered cells for the life of the recipient. However, HSCs have a low level of amphotropic receptor and are poorly transducible(12). The HSC remains, therefore, an important but elusive target.

The broad range of cell types possessing the amphotropic receptor, known to be a phosphate symport, limits the target-specific utility of these vectors in the *in vivo* approach. Using different viral envelope proteins that recognize different receptors (for example, the vesicular stomatitis virus (VSV)-G protein or the gibbon ape leukaemia virus (GALV) envelope protein) can vary the range of cells that can be transduced, but still does not provide much specificity. The difficulty is that, because retroviral vectors cannot be generated at a high titre (amphotropic vectors appear to be limited to 1 x 10^7 colony-forming units (CFU) per ml and VSV-G pseudotyped vectors to 1 x 10^9 CFU per ml), it is not possible to get a large number of vector particles to the desired cell type *in vivo*. The viral particles would bind to many cells they encounter and, therefore, would be diluted out before reaching their target (other issues, such as complement-mediated lysis, will be discussed later). The problem can be quantified. The human body contains about 5 x 10^13cells. If a 100 ml sample of retroviral vector were given to a patient, that would be about 1 x 10^9 active vector particles. Even if every vector particle were 100% efficient at infection, only 1 cell in 50,000 would be transduced. What is needed is a retroviral particle that will preferentially bind to its target cell and can be manufactured at a high titre.

Efforts to target specific cell types have centered on attempts to engineer the natural retroviral envelope protein. The envelope protein has two functions: binding to its receptor (by the surface (SU) moiety) and enabling the entry of the viral nucleoprotein core (carried out primarily by the transmembrane (TM) moiety). The SU protein binds to its receptor on the target cell surface and, as a result, the SU/TM complex undergoes a conformational change that allows fusion of the viral and cellular membranes, followed by entry of the viral core (which carries the virus's genetic information) into the target cell’s cytoplasm (Fig. 1).

Two broad approaches to providing target cell specificity have been followed. First, the natural receptor-binding domain of the SU protein has been replaced with a ligand or single-chain antibody that recognizes a specific cell surface receptor (13,14). A wide range of receptors have been targeted, but the difficulty is that even though specific binding can be obtained between the engineered vector and the target cell receptor, gene transfer has been unacceptably low in all these experiments. The reason is clear. The retroviral envelope protein is thought to be a trimer with a complex quaternary structure (15). When the natural receptor-binding domain is replaced by a foreign sequence, the whole structure of the envelope protein is altered. The result is that the natural post-binding conformational. change that leads to the fusion of the virus with the cell membrane does not occur. Without fusion, core entry and gene transfer do not take place efficiently.

Engineering the receptor-binding domain of SU while maintaining the ability of the envelope protein to carry out core entry will require a better understanding of the structure-function relationships within the envelope protein complex. This understanding has been enhanced by the recent publication of the three-dimensional structure of the receptor-binding domain of the murine ecotropic (Friend strain) SU protein (16). It should now be possible to engineer ligands into very specific sites in the SU protein with a higher probability of maintaining the functional properties of the envelope protein for core entry.

Other structure-function studies of the retroviral envelope protein are also contributing to our understanding of how to obtain efficient core entry after binding. The three-dimensional structure of a portion of the Moloney ecotropic retroviral TM protein was published last year. Recently it has been shown that the separate monomers in the predicted trimeric structure of the envelope can cross-talk with each other (17). In other words, separate monomers, each of which is defective, can complement each other to provide an active trimeric envelope. Using this technique it has been possible to define separate functional domains in the TM protein (18). As the complete three-dimensional structure and functional domains of the envelope protein become known, constructing retroviral vectors that are able to target specific cells with high efficiency should be possible.

Progress has been made using a second broad approach to targeting that could be called 'tethering'. Although several creative systems have been designed, the most successful approach at present appears to be insertion of a ligand that recognizes an extracellular matrix (ECM) component into a part of the SU protein that does not disturb the natural receptor-binding domain. This tethering concentrates the vector in the ECM in the vicinity of the target cells. Receptor binding and core entry can then occur through the natural envelope-receptor mechanism. Two ligands that appear particularly useful for tethering are those specific for fibronectin(19) for collagen(20). Fibronectin is present in normal

ECM and exposed collagen is present in areas of damage, for example after wound injury as in the cardiovascular system after angioplasty.

Transduction of non-dividing cells. Although the inability of MULV-based retroviral vectors to transduce non-dividing cells is very useful in some situations, for example when a toxin gene is being inserted into dividing cancer cells and not into the normal non-dividing cells (see below under 'Clinical studies'), there are many situations where one would want to insert a therapeutic gene into normal non-dividing cells. Many potential target cells are not actively dividing *in vivo*; only certain blood cells (not the stem cell) and the cells lining the gastrointestinal tract are continually in division. Lentiviruses (such as HIV-1) are able to infect non-dividing cells, but vectors constructed from these viruses raise concerns over safety because of the possibility that recombination could produce a pathogenic virus. Attempts to transfer into murine retroviral vectors the specific signals from HIV that allow transduction of non-dividing cells have not been successful. Recently it has become possible to use just 22% of the HIV genome (which does not include any of the genes that cause pathology) in a retroviral vector (21,22). The chances of recombination have been further reduced by the use of a non-HIV envelope protein. This hybrid system holds great promise for providing the option of transducing non-dividing cells *in vivo* in a safe manner. Another RNA viral system being developed is based on the human foamy virus (23). These vectors are able to transduce a broad range of cell types, are not inactivated by human serum, and may be able to transduce some non-dividing, as well as dividing, cells.

Improving gene expression. Assuming that efficient gene transfer can be developed, the next issue is long-term, stable gene expression at an appropriate level (6). This is perhaps the greatest shortcoming of present vectors. Although gene expression is being discussed here under retroviral vectors, the topic applies to gene transfer vectors of all types.

Several factors are involved in maintaining the stable expression of genes after their transfer. First, the regulatory sequences that control gene expression often do not remain active. There is a tendency for the cell to recognize foreign promoters (particularly viral promoters such as simian virus 40 (SV40) and cytomegalovirus (CMV)) and inactivate them (by methylation or other mechanisms). The role of lymphokines, cytokines and other growth factors in maintaining gene expression is also poorly understood. Second, even if the gene stays active within the cell, the cell often dies. The immune system is designed to recognize and eliminate foreign gene products and cells that produce a foreign protein. All viral genes are eliminated from retroviral vectors, and so immune recognition of viral proteins (except for those, such as capsid proteins, that are packaged into the viral particle itself) is not an issue (but see the discussion of adenoviral vectors below). Nonetheless, the immune system is still likely to recognize a new or modified protein produced by the therapeutic gene; a newly synthesized normal protein will appear abnormal to an immune system that has never been exposed to it.

Use of a cell's own *cis*-regulatory DNA sequences will probably provide more stable long-term gene expression than can be obtained with viral promoters, but identifying all the components of a gene's regulatory system can be difficult. As an extreme case, the regulatory sequences involved in the proper regulation of the haemoglobin (B-globin) genes are spread over nearly 100 kb. Because a retroviral vector can only accommodate 6-8 kb of sequence, regulatory sequences may need to be truncated to their minimal essential length before being incorporated into such vectors. Even when the natural regulatory elements are used, they may not function correctly without the proper signals and feedback mechanisms that normally operate in the appropriate cellular milieu. For example, the insulin enhancer/promoter still cannot direct regulated expression when delivered to fibroblasts. Again, this emphasizes the need to develop vectors that are capable of gene transfer to specific cell types.

There is steady progress on these fronts, but long-term, stable, appropriate-level gene expression *in vivo* in a range of cell types is still to be accomplished. Once these hurdles are cleared, the next goal will be to achieve gene expression that can be regulated. Many important target genes, such as that for insulin, are not expressed at the same level all the time, but respond to physiological signals within the body. The goal is to use regulatory sequences that respond to the body's own physiological signals (so that inserted therapeutic genes can function the way that normal endogenous genes do) or to drugs that can be used to control the level of gene activity. In some cases, only low levels of essentially unregulated expression may be beneficial (for example, in haemophilia or adenosine deaminase (ADA) deficiency), whereas in other cases tight regulation may be required (for example, for insulin production in diabetes).

Manufacturing the vector. Although consideration of how a pharmaceutical company would be able to manufacture millions of doses of a gene-therapy vector was irrelevant a decade ago, this has now become a real issue. Retroviral vectors are biological agents: they can only be made by living cells. Biological systems are not the easiest systems in which to carry out good manufacturing practice (GMP) and quality assurance/quality control (QA/QC) procedures mandated by the Food and Drug Administration (FDA), as manufacturers of vaccines have learned.

One of the major concerns with retroviral vectors is the possibility that a replication-competent retrovirus (RCR) could arise during the manufacturing process. Because retroviral vectors are produced in packaging cells that contain a packaging-defective viral genome, and because retroviruses have a high propensity for recombination, this possibility is always present. Furthermore, as every mammalian cell contains endogenous retroviruses, additional viral sequences could be incorporated into the RCR, perhaps producing a pathogenic virus.

Another potential problem results from the ability of retroviral vectors to integrate randomly into host cell DNA. For example, a vector might insert itself into a tumour suppressor gene, thereby increasing the propensity of the cell to become cancerous. The only example of unintentionl tumour production in a retroviral gene transfer experiment in large animals was published in 1992; three cases of lymphoma were reported among ten rhesus monkeys whose bone marrow had been destroyed by irradiation and who were then transplanted with haematopoietic stem cells that had been exposed to a large number of RCR as well as the experimental vector (24). It was shown that the cancers resulted from integration of an RCR (not of the retroviral vector), were clonal events and developed only after a long period (6-7 months) of retroviraemia.

The subject of RCR production and safety as well as of potential tumour production was extensively analyzed in a report to the NIH Recombinant DNA Advisory Committee (RAC and the FDA (25). The conclusion was that the current QA/QC procedures required by the FDA make it exceedingly unlikely that any patient could receive sufficient RCR to produce either a retroviraemia or a malignancy. However, the manufacturing and testing process to ensure this degree of safety is complex and expensive.

As the goal of present research is the production of a gene therapy vector that can be injected directly into the body (just like penicillin or insulin), additional problems must be considered. For example, mouse packaging cells produce retroviral vectors that are destroyed by human complement. Although this sensitivity makes the vector particles "safer", it does markedly reduce their half-life *in vivo* and the efficiency of gene transfer. The major component of this sensitivity arises from the presence of unique sugar groups on viral glycoproteins produced in the murine packaging cells that make the viral particles sensitive to human complement. Either the vector particles produced in mouse cells must be engineered to avoid the human complement system, or the vector needs to be made in a non-murine packaging cell line that can provide the viral particles with appropriate sugar groups on their surface. However, as mentioned above, essentially all mammalian cells have their own endogenous retroviruses that could recombine with the vector to produce a new, potentially pathogenic, RCR; many of these endogenous viruses are still unknown. Although any cell line is suspect, the use of primate or human cells as packaging cells raises the greatest safety concerns in this regard. Human packaging cells can, however, be engineered to be very safe. For example, the ProPak cell line (26), which has the viral *gag-pol* genes on a separate DNA construct from the *env* gene (producing a ‘split’ packaging cell line) as well as other safety features, is certainly safer than the murine packaging cell line PA317, which is used for most of the present retroviral vector clinical trials.

These issues are resolvable, but it will take several years of product development to develop a cost-effective manufacturing system that will produce safe, efficient gene-therapy vectors on a sufficient scale to allow worldwide marketing. Although a non-viral delivery system that avoids many of these problems may be the gene-therapy vector of the future (see discussion below under 'Non-viral vectors'), the many present and future clinical protocols using retroviral vectors require that the manufacturing issues of safety and efficiency be solved.

Vectors based on DNA viruses

Adenoviral vectors. The DNA virus used most widely for *in situ* gene transfer vectors is the adenovirus (specifically serotypes 2 and 5). Adenoviral vectors have several positive attributes: they are large and can therefore potentially hold large DNA inserts (up to 35 kb, see below); they are human viruses and are able to transduce a large number of different human cell types at very high efficiency (often reaching nearly 100~% *in vitro*); they can transduce non-dividing cells; and they can produced at very high titres in culture. They have been the vector of choice cells for several laboratories trying to treat the pulmonary complications of cystic fibrosis, as well as for a variety of protocols attempting to treat cancer.

Adenoviral vectors have certain drawbacks, however. First-generation vectors were deleted for the early region 1 (E1) functions in order to render them replication-defective. In addition, these vectors were deleted in the E3 region in order to create space for the insertion of transgenes. The E3 region, as discussed below, functions to suppress the host immune response during virus infection, but is not required for replication or packaging *in vitro*. Vectors with El and E3 deleted elicited strong inflammatory and immune responses (27). This is thought to be a consequence of "leak" expression of adenoviral proteins in the transduced cells because these first-generation vectors retain most of the viral genome. It was hoped that a weaker immune response would result if additional viral genes were deleted. Thus vectors with the deletion of E1 coupled with the deletion of other essential early genes, E2a and/or E4 (28, 29), or vectors with all of the viral genes deleted (so-called 'gutless' vectors(30-32) have been constructed and tested in animals. There have been conflicting reports regarding the immunogenicity, stability of gene expression, and persistence *in vivo* of gutless vectors(33).In fact,these properties may differ depending on the exact vector design, the tissue type that the vector is introduced into, and the nature of the transgene insert. In particular, the gutted vectors offer the possibility of introducing up to 35 kb of genomic sequences, and it has been suggested that inclusion of nuclear matrix attachment regions might facilitate long-term gene expression and persistence of the vector sequences.

Deleting more and more viral genes may not always be advantageous because some of these genes may have beneficial attributes, for example suppressing an immune response against the vector. Their removal could increase the rate at which the vector is eliminated. As an example, the E3 region encodes a protein of relative molecular mass 19K that protects the virus, and presumably the engineered cells, from host immune surveillance (34). Various effector mechanisms may be involved in viral vector clearance (35). In addition, *cis*-acting sequences may exist that help maintain the stability of the adenoviral genome in the cell. As with drug trials, results in animals (even in primates) have not always reflected what happens in patients. Vectors that produce inflammatory responses in primates may not do so in human patients, and the opposite situation is probably also likely. Recently, the first 'true' phase I gene therapy clinical trials have begun: normal volunteers have been tested with intradermal injection (and now by intrabronchial infusion) of adenoviral vectors in order to determine the immunological response to adenoviral vectors in human beings.

By engineering the correct combination of viral genes (incorporating immunosuppressive genes, perhaps from various sources, while deleting immune-stimulating gene products and reducing, if possible, the immunogenicity of viral capsid proteins), it is likely that adenoviral vectors can be generated that have low toxicity, that do not generate an immune response, and that, therefore, can be given repeatedly. The latter point is important because adenoviral vectors do not integrate and they survive in the cell for a limited time (although in non-dividing cells this may be for an extended period).

The ability to administer the vector repeatedly will be critical in many treatment protocols, for example in those for haemophilia and cystic fibrosis. Although it would dearly be optimal to engineer vectors that do not elicit an immune response, an interim solution could be to use transient immunosuppression of the patient to allow repeated administration of vectors. Another approach is to blockade costimulatory interactions required for an immune response to an antigen, thereby transiently 'blinding' the immune system during vector administration and making repeat administration possible.

Adeno-associated viral vectors. Another DNA virus used in clinical trials is the adeno-associated virus (AAV). This is a non-pathogenic virus that is widespread in the human population (about 80% of humans have antibodies directed against AAV). Initial interest in this virus arose because it is the only known mammalian virus that shows preferential integration into a specific region in the genome (into the short arm of human chromosome). As the virus does not produce disease, its insertion site appears to be a 'safe' region in the genome. It would be useful, therefore, to engineer the sequences that dictate this site-specific insertion into gene therapy vectors. Unfortunately, the present AAV vectors appear to integrate in a nonspecific manner (36), although it has been suggested that vectors could be designed that retain some specificity (37).

Even though integration site specificity has not been achieved, AAV vectors have been shown transduce brain, skeletal muscle, liver and possibly CD34+ blood cells efficiently (2,38-40).There are several drawbacks, however: some cells require a very high multiplicity of infection (the number of viral particles per cell required to achieve transduction); the AAV genome is small, only allowing room for about 4.8 kb of added DNA; and the production of viral particles is still very labour intensive because efficient packaging cells have not yet been developed. However, these vectors hold promise and appear to be safe. Furthermore, AAV may be capable of integrating into non-dividing cells, although again this desirable attribute of the wild-type virus appears to be lost from the vectors, which can enter non-dividing cells but remain in an episomal state until cell division occurs.

Other DNA virus-based vectors

Other DNA viruses are being studied as possible gene-therapy vectors for specific situations. For example, herpes simplex virus (HSV) vectors have a propensity for transducing cells of the nervous system (41,42),as well as several other cell types. A stripped-down version of the HSV, called an amplicon, may have certain advantages, particularly when combined with components from other viral systems (43). A number of other DNA virus vectors are under study including poxviruses. Several investigators are examining replication-competent, or attenuated, viral vectors (both DNA and RNA). In addition, hybrid systems have been reported where an adenoviral vector is used to carry a retroviral vector into a cell that is normally inaccessible to retroviral transduction (44).

Non-viral vectors

Although viral systems are potentially very efficient, two factors suggest that non-viral gene delivery systems will be the preferred choice in the future: safety, and ease of manufacturing. A totally synthetic gene-delivery system could be engineered to avoid the danger of producing recombinant virus or other toxic effects engendered by biologically active viral particles. Also, manufacturing a synthetic product should be less complex than using tissue culture cells as , bioreactors, and QA/QC procedures should be simplified. The reader is referred to the review on non-viral vectors entitled 'Drug delivery and targeting' by Robert Langer.

Clinical studies

At present over 300 clinical protocols have been approved. Detailed information is available on the 232 protocols that had been approved in the USA as of 3 February 1998(45,Table1)

Only one phase III and several phase 11 clinical trials are now underway; all rest of the approved gene therapy clinical protocols are for smaller phase I/II trials. Genetic Therapy Inc./Novartis is carrying out the phase-III clinical trial. The target disease is glioblastoma multiforma, a malignant brain tumour (46). The rationale is to insert a gene capable of directing cell killing into the tumour while protecting the normal brain cells. The retroviral vector used (G1TkSvNa) contains the neomycin-resistance gene as a selective marker and the herpes simplex thymidine kinase (HSTK) gene. The actual material injected into the tumour mass is a mouse producer cell line (PA317) which generates retroviral particles carrying the G1TkSvNa vector. As the only dividing cells in the area of a growing brain tumour are the tumour cells and cells of the vasculature supplying blood to the tumour, and retroviral vectors only transduce dividing cells, the only cells to receive the vector should be the cells of the tumour and its blood vessels. The viral HSTK can add a phosphate group to a non-phosphorylated nucleoside, whereas the endogenous human thymidine kinase cannot. Therefore, when an abnormal nucleoside, such as the drug ganciclovir, is given to the patient, only the cells expressing the HSTK gene will phosphorylate the drug, incorporate it into their DNA synthesis machinery and be killed.

In the current phase III clinical trial, mouse producer cells making vector particles carrying the HSTK gene are inoculated into residual tumour and peritumour areas following tumour resection. After Mays, the patient is treated with ganciclovir. In theory, the tumour cells that have been transduced with the vector containing the HSTK gene will phosphorylate ganciclovir; the ganciclovir triphosphate then blocks the DNA synthesis machinery and kills the cells.

In fact, at least four distinct mechanisms contribute to tumour cell death in this protocol. First is the direct effect of phosphorylated ganciclovir on the thetransduced tumour cells; second is the 'bystander' effect in which toxic agents (ganciclovir triphosphate) pass into neighbouring cells through gap junctions and kill them; third, is the local inflammatory effect caused by the injected mouse cells; and fourth is a systemic immune response. The phase III trial includes a total of more than 40 centres in North America and Europe and is scheduled to enroll a total of 250 patients. By the end of December 1997 over 200 patients had been enrolled.

Several phase II trials are underway testing gene-therapy vectors as 'vaccines', either against cancer (48) or against AIDS (49). Vical has two active phase II trials using a plasmid containing the gene for the HLA-B7/B2-microglobulin protein formulated with cationi16 lipids. One trial is for metastatic malignant melanoma and the other for head and neck squamous cell carcinoma. The concept is that an HLA gene (such as B7) that the tumour does not express is injected into the tumour mass and that expression of this foreign antigen should stimulate the immune system to react against the cancer. The data so far suggest that the immune system not only develops a response against the B7 antigen but also to other ant* on the tumour cells, thereby resulting in an immune attack on non-transduced tumour cells (58). Viagen/Chiron has completed a phase II trial of about 200 patients over 2 years in which a retroviral vector encoding the *env* and *rev* gene segments of the HIV- I (IIIB) strain is injected intramuscularly to induce augmented anti-HIV cytotoxic T-cell responses as a treatment for AIDS. Unfortmately, determination of the efficacy of this treatment was made impossible by the advent of triple drug therapy for HIV infection, but no evidence of toxicity was seen.

Finally, a comment on the original adenosine deaminase (ADA) deficiency gene-therapy trial (1,51). ADA deficiency is a rare genetic disorder that produces severe immunodeficiency in children. Starting in 1990, gene-corrected autologous T lymphocytes were given to two girls suffering from this disease. Both girls are doing well and continue to lead essentially normal lives. Patient 1 (A.D.) received a total of 11 infusions, the last being in the summer of 1992. Her total T-cell level and her level of transduced T cells have remained essentially constant for the past 5 1/2 years. She contracted chickenpox in late 1996 and experienced the same clinical course as would have been expected for any normal 10-year-old. Both she and patient 2 (C.C.) continue to receive polyethylene glycol (PEG)-ADA. Although both girls have gene-engineered T lymphocytes in their circulation after more than 7 years, no definitive conclusion can be drawn as to the relative roles of PEG-ADA and gene therapy in their excellent clinical course.

Ethical issues

Somatic cell gene therapy for the treatment of serious disease is now accepted as ethically appropriate. Indeed, it is so well accepted, and the side effects from gene-therapy protocols have been so minimal, that the danger now exists that genetic engineering may be used for non-disease conditions, that is for functional enhancement or 'cosmetic' purposes. The first Gene Therapy Policy Conference organized by the NIH RAC focused on this issue in September 1997. The conclusion was that enhancement engineering is about to take place, and could slip through the regulatory process if RAC and the FDA (and similar organizations in other countries) are not vigilant. As an example, a US biotechnology company has developed the technology for transferring genes (specifically the tyrosinase gene) into hair follicle cells (52). They are now looking for genes that promote hair growth with the clinical objective of reversing the hair loss that occurs after chemotherapy in cancer patients. The application to the FDA for product licensing would list chemotherapy-induced alopecia as the product indication. The risk-benefit analysis here would be very favorable. However, once a product is licensed for any indication, it can be prescribed by physicians for any 'off-label' use that is felt by the physician to be clinically justified. The result could be millions of balding men receiving gene therapy to treat their hair loss. The conference concluded that the FDA should use a risk-benefit analysis that takes into account the extensive off-label usage for cosmetic reasons that could take place.

Using genetic engineering to treat baldness is not a major issue in itself, of course. But this is just one example of how our society is moving towards a slippery slope where genetic engineering might very well be used for a broad range of enhancement purposd'g, including larger size from a growth hormone gene, increased muscle mass from a dystrophin gene and so on. If we knew that there would be no long-term negative effects of genetic engineering, then widespread, or even frivolous, use of genetic engineering technology might not be detrimental. But just as with nuclear energy, pesticides and fluorocarbons, we as a society tend to see the benefits but are caught off guard by the bad effects of our powerful new technologies. What society wants to do 100 years from now with regards to genetic engineering is their business, but it is our duty to begin the era of genetic engineering in as responsible a manner as possible. Until we have learned about the long-term effects of somatic cell gene therapy in the treatment of disease, we should not use this technology for any other purpose than where it is medically indicated (53).

In utero somatic gene therapy of the fetus will be undertaken in the foreseeable future. The same care should be exercised here as with somatic cell gene-therapy protocols for adults, children and newborns. So long as only serious disease is targeted and the risk-benefit ratios for both mother and the fetus are acceptable, *in utero* gene therapy should be ethically appropriate (54). Germline gene therapy should not be attempted at this time for the reasons outlined elsewhere (55).

A situation with the potential for real abuse of the new technologies would be the combination of cloning and genetic engineering. This combination has already been achieved in sheep where single cells have been obtained from fetal fibroblasts, transduced with a gene (human factor IX), and the gene-engineered cells grown into living sheep producing human factor IX (56) . Attempts to use such techniques to produce genetically engineered humans would provoke an even greater ethical storm than the present suggestion by a Chicago scientist to clone humans.

The future

The ultimate goal of gene-therapy research is the development of vectors that can be injected, will target specific cells, will result in safe and efficient gene transfer into a high percentage of those cells, will insert themselves into appropriate regions of the genome (or will persist as stable episomes), will be regulated either by administered agents or by the body's own physiological signals, will be cost-effective to manufacture and will cure disease. As the number of target cells may be in the billions, very high efficiency of gene transfer and the injection of a large number of gene-therapy vectors may be necessary. How soon can we expect significant progress in each of these areas?

The next 5 years should bring the first successes for gene therapy, that means statistically significant data that a gene-therapy protocol results in significant improvement in the clinical condition of patients. Within this time frame the first vectors that can target specific tissues should begin clinical trials and tissue-specific gene expression should have made its way into clinical trials.

In a time frame of 5-15 years from now, I expect that the number of gene-therapy products will begin to increase exponentially, coinciding with the enormous increase in characterized genes as a result of the Human Genome Project. The first injectable vectors'will reach clinical trials and efficient tissue-specific gene transfer will be available in a few cases. It will probably take longer to develop site-specific integration, efficiently regulated genes and the correction of genes *in situ* by means of homologous recombination. Beyond this, our imagination is the limit.

For many gene-therapy applications in the future, it is probable that a synthetic hybrid system will be used that incorporates engineered viral components for target-specific binding and core entry, immunosuppressive genes from various viruses and some mechanism that allows site specific integration, perhaps utilizing AAV sequences or an engineered retroviral integrase protein. In addition, regulatory sequences from the target cell itself will be utilized to allow physiological control of expression of the inserted genes. AU these components would be assembled *in vitro* in a liposome-like formulation with additional measures taken to reduce immunogenicity such as concealment by PEG.

Conclusions

Gene therapy is a powerful new technology that still requires several years before it will make a noticeable impact on the treatment of disease. Several major deficiencies still exist including poor delivery systems, both viral and non-viral, and poor gene expression after genes are delivered. The reason for the low efficiency of gene transfer and expression in human patients is that we still lack a basic understanding of how vectors should be constructed, what regulatory sequences are appropriate for which cell types, how *in vivo* immune defences can be overcome, and how to manufacture efficiently the vectors that we do make. It is not surprising that we have not yet had notable clinical successes. Nonetheless, the lessons we are learning in the clinic are invaluable in illuminating the problems that future research must solve. Despite our present lack of knowledge, gene therapy will almost certainly revolutionize the practice of medicine over the next 25 years. In every field of medicine, the ability to give the patient therapeutic genes offers extraordinary opportunities to treat, cure and ultimately prevent a vast range of diseases that now plague mankind.

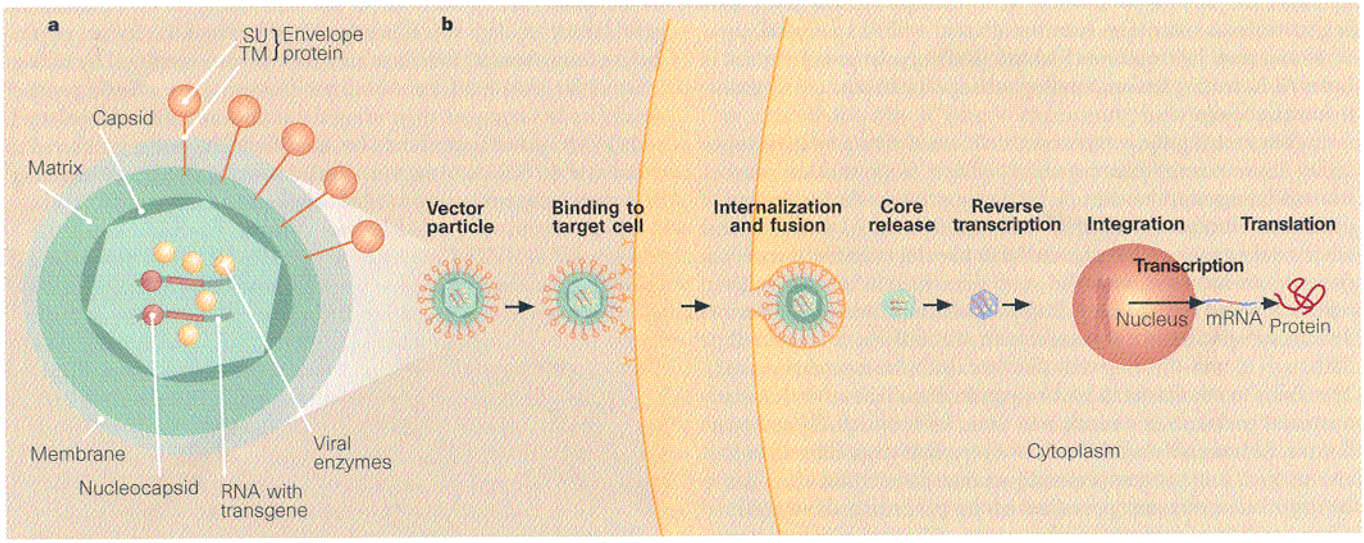

Figure 1 The protocol for retroviral vectors. **a,** Diagram of a retroviral vector. The vector particle is covered by a membrane (derived from the membrane of the cell from which the viral particle budded). Anchored in the membrane is the envelope protein, which is composed of two parts: the SU (surface) protein, which is responsible for binding to the receptor on the target cell, and the TM (transmembrane) protein, which passes through the membrane into the viral matrix and is involved in the fusion step. Beneath the viral membrane is the matrix protein and deeper still is the viral core, which is composed of a surrounding capsid within which are two identical strands of RNA together with the nucleocapsid protein and the viral enzymes (protease, polymerase and integrase). In a retroviral vector the viral genes have been replaced by a transgene. **b,** Diagram of a retroviral vector transducing a target cell. First, the vector particle binds to its receptor on the target cell by means of its SU envelope protein. The particle is then internalized into the cytoplasm of the cell, encased inside an endosome. The envelope protein initiates fusion of the viral membrane with the endosomal membrane, causing the viral core to be released into the cytoplasm. Reverse transcription takes place within the core, which results in the RNA being copied into a double strand of DNA. The double-stranded viral DNA then enters the nucleus, integrates into the chromosomal DNA, and is transcribed. Because the viral genes have been replaced by a transgene, only the protein product of the transgene is made instead of new viral particles.

Box 1 The three categories of somatic cell gene therapy

- The first is *ex vivo*, where cells are removed from the body, incubated with a vector and the gene-engineered cells are returned to the body. This procedure is usually done with blood cells because they are the easiest to remove and return.
- The second is *in situ*, where the vector is placed directly into the affected tissues. Examples are the infusion of adenoviral vectors into the trachea and bronchi of patients with cystic fibrosis, the injection of a tumour mass with a vector carrying the gene for a cytokine or a toxin, or the injection of a vector carrying a dystrophin gene directly into the muscle of a patient with muscular dystrophy.
- The third is *in vivo*, where a vector could be injected directly into the bloodstream. There are no clinical examples of this third category as yet, but if gene therapy is to fulfil its promise as a therapeutic option, *in vivo* injectable vectors must be developed.

Table 1 Disease targets and gene-therapy protocols

(a) Types of gene therapy clinical protocols*

Type	Number	Percentage of total
Therapy	200	(86%)
Marker	30	(13%)
Non-therapeutic†	2	(1%)
Total	232	(100%)

(b) Disease targets for therapeutic gene therapy clinical protocols

Target	Number	Percentage of total
Cancer	138	(69%)
Genetic diseases	33	(16.5%)
CF	16	
Other‡	17	
AIDS	23	(11.5%)
Other§	6	(3%)
Total	200	(100%)

* Roughly 60% of all protocols use retroviral vectors, 20% use non-viral delivery systems, 10% use adenoviral vectors and the remainder use other viral vectors.

† A 'non-therapeutic' protocol means a non-therapeutic portion of a non-gene-therapy clinical protocol.

‡ These 17 include 12 other monogenic diseases.

§ The five 'other' are: peripheral artery disease, rheumatoid arthritis, arterial restenosis, cubital tunnel syndrome and coronary artery disease (2).

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1. *Blaese, R. M. et at. T lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years. Science 270,475-480 (1995).*

2. Kay, M. A., Liu, D. & Hoogerbrugge, P. M. Gene therapy. *Proc. Nad Acad. Sci.* USA 94, 12744-12746 (1997).

3. Verma, I.M. & Somia, N. Gene therapy: promises, problems and prospects. *Nature* 389,239-242 (1997).

4. Havenga, M., Hoogerbrugge, P, Valerie, D. & van Es, H. H. G. Retroviral stem cell gene therapy. Stem Cells 15,162-179 (1997).

5. Dass, C.R. et *al.* Cationic liposomes and gene therapy for solid tumors. *Drug Delivery* 4, 151-165 (1997).

6. Miller, N. & Whelan, 1. Progress in transcriptionally targeted and regulatable vectors for genetic therapy. *Hum. Gene Ther.* 8,803-815 (1997).

7. Cosset, R-L. & Russell, S. J. Targeting retrovirus entry. Gene net. 3,946-956 (1996).

8. Cristiano, R. J. & Curiel, D. T. Strategiq to accomplish gene delivery via the receptor-mediated enclocytosis pathway. Cancer Gene Ther. 3,49-5~ (1996).

9. Brenner, M. Gene marking. Hum. Gene Ther. 7,1927-1936 (1996).

10. Schmerle, B. S. & Groner, B. Retroviral targeted delivery. Gene Ther. 3, 1069-1073 (1996).

11. Anderson, W E Prospects for human gene therapy. Science 226,401-409 (1984),

12. Orlic, D. et al. The level of mRNA encoding the arnphotropic retrovirus receptor in mouse and human hematopoietic stem cells is low and correlates with the efficiency of retrovirus transcluction. Proc. Natl Acad. Sci. USA 93, 11097-11102 (1996).

13. Salmons, B. & Gunzburg, W. H. Targeting of retroviral vectors for gene therapy. *Hum. Gene Ther.* 4, 129-141 (1993).

14. Kasahara, N. A., Dozy, A. M. & Kan, Y. W. Tissue-specific targeting of retroviral vectors through hgandreceptor interactions. Science 266, 1373-1376 (1994).

15. Fass, D., Harrison, S. C. & Kim, R S. Retrovirus envelope domain at 1.7 A resolution. Nature Struct. Biol. 3,465-469 (1996).

16. Fass, D. et aL Structure of a marine leukemia virus receptor-binding glycoprotein at 2.0 angstrom resolution. Science 277, 1662-1666 (1997).

17. Zhao, Y., Lee, S. & Anderson, W. E Functional interactions between monomers of the retroviral envelope protein complex. 1. ViroL 71, 6967-6972 (1997).

18. Zhao, Y. et al. Functional domains in retroviral transmembrane protein. J. Viral. (in the press).

19. Hanenberg, H. et al. Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. Nature Med. 2, 876-882 (1996)

20. Hall, F. L. et al. Targeting retroviral vectors to vascular lesions by genetic engineering of the MoMLV gp70 envelope protein. Hum. Gene Ther. 8, 2183-2192 (1997).

21. Zufferey, R. et al. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nature Biotechnol. 15,871-875 (1997).

22. Kafti, T et al. Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. Nature Genet. 17, 314-317 (1997).

23. Russell, D. W. & Miller, A. D. Foamy virus vectors. J. ViroL 70, 217 -222 (1996).

24. Donahue, R. E. et aL Helper virus induced T cell lymphoma in nonhuman primates after retroviral mediated gene transfer. *1.* Exp. Med. 176, 1125-1135 (1992).

25. Anderson, W. E, McGarrity~ G. J. & Moen, R. C. Report to the NIH Recombinant DNA Advisory Committee on murine replication-conapetent retrovirus (RCR) assays. Hum. Gene Ther. 4,311-321 (1993).

26. Forestell, S. P. et al. Novel retroviral packaging cell lines: complementary tropisms and improved vector production for efficient gene transfer. Gene Ther. 4,600-610 (1997). '

27. Ali, M., Lernoine, R. & Ring, J. A. The use of DNA viruses as vectors for gene therapy. Gene Ther. 1, 367-384 (1994).

28. Gao, G.-P., Yang, Y. & Wilson, J. M. Biology of adenovirus vectors with El and E4 deletions for liver-directed gene therapy. *1.* Virol. 70, 8934-8943 (1996).

29. Dedieu, L-F. et al. Long-term gene delivery into the livers of immunocompetent mice with E1/E4-defective adenoviruses. 1. Viral. 71, 4626-4637 (1997).

30. Haecher, S. E. et aL In vivo expression of full-length human dystrophin from adenoviral vectors deleted of all viral genes. Hum. Gene Ther. 7,1907-1914 (1996)

31. Lieber, A. et al. Recombinant adenoviruses with large deletions generated by cre-mediated excision exhibit different biological properties compared with first-generation vectors in vitro and in vivo. *1.* Viral. 70, 8944-8960(1996).

32. Chen, H. H. et aL Persistence in muscle of an adenoviral vector that lacks all viral genes. Proc. Natt Acad. Sci. USA 94, 1645-1650 (1997).

33. Kaplan, 1. M. et al. Characterization of factors involved in modulating persistence of transgene expression from recombinant adenovirus in the mouse lung. *Hum. Gene Ther.* 8,45-56 (1997).

34. Ginsberg, H. S. et aL Role ofearly region 3 (E3) in pathogenesis ofadenovirus disease. Proc. NatlAcad. Sci. USA 86, 3823-3827 (1989).

35. Elkon, K. B. et al. Tumor necrosis factor a plays a central role in immune-mediated clearance ofadenoviral vectors. Proc. Natl Acad. Sci. USA 94, 9814-9819 (1997).

36. Kearns, W. G. et al, Recombinant adeno-associated virus (AAV-CFTR) vectors do not integrate in a sitespecific fashion in an immortalized epithelia] cell line. Gene Ther. 3, 748-755 (1996).

37. Linden, R. M. & Berns, K. 1. Site-specific integration by adeno-associated virus: a basis for a potential gene therapy vector. Gene Ther. 4, 4-5 (1997).

38. Miller, A. D. Putting muscle to work for gene therapy. Nature Med. 3, 278-279 (1997).

39. Yjao, X., Li, J. & Samulski, R. J. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector, *1.* Viral. 70, 8098-8108 (1996).

40. Fisher, K. J. et al. Recombinant adeno-associated virus for muscle directed gene therapy. *Nature Med. 3,* 306-312 (1997).

41. Breakefield, X. 0. & DeLuca, N. A. Herpes simplex virus for gene delivery to neurons. New Biol. 3, 203-218 (1991).

42. Fink, D. J. & Glorioso, J. C. Engineering herpes simplex virus vectors for gene transfer to neurons. Nature Med. 3,357-359 (1997).

43. Jacoby, D. R., Fraefel, C. & Breakefield, X. 0. Hybrid vectors: a new generation of virus-based vectors designed to control the ceHular fate of delivered genes. *Gene Ther.* 4, 1281-1283 (1997).
44. Bilbao, *G. et aL* Adenoviral/retroviral vector chimeras: a novel strategy to achieve high-efficiency stable transcluction in vivo. *FASEB 1.* 11, 624-634 (1997).

45. ORDA Report: Human Gene Therapy Protocols (2/3/98) (Office of Recombinant DNA Activities, NIH, Bethesda, MD, 1998).

46. Ram, Z. et al. Therapy of malignant brain tumors by intratumoral implantation of retrovirod vector-producing cells. *NatureMed. 3,1354*-1361 (1997).

47. Oldfield, E. H. et A Gene therapy for the treatment of brain tumors using intra-tumoral transduction with the thymidine kinase gene and intravenous ganciclovir. Hum. *Gene Ther.* 4,39-69 (1993).

48. Stopeck, A. T. *etal.* Phase I study ofdirect gene transfer ofan allogeneic histocompatibility antigen, HLAB7, in patients with metastatic melanoma. *1. Clin. Oncol.* 15, 341-349 (1997).

49. Haubrich, R. & McCutchan, J. A. An open label, phase 1/11 clinical trial to evaluate the safety and biological activity ofHrV-IT (V) (HIV- 1111B-'retroviral vector) in HTV- I-infected subjects. Hum. *Gene Ther.* 6, 941-955(1995).

50. Nabel, G. J. *et al* Immune response in human melanoma after transfer of an allogeneic class I major histocompatibflity complex gene with DNA-liposome complexes. Proc. Natl Arad. Sci. USA 93, 15388-15393 (1996).

51. Anderson, W E Human gene therapy. Science 256,808-813 (1992).

52. Hoffman, R. M. et al. The feasibility of targeted selective gene therapy of the hair follicle. Nature *Med. 1,* 705-706 (1995).

53. Anderson, W F. Human gene therapy: why draw a line? *1. Med. Philos.* 14, 681-693 (1989).

54. Fletcher, J. C. & Richter, G. Human fetal gene therapy: moral and ethical questions. *Hum. Gene Ther.* 7, 1605-1614 (1996).

55. Fletcher, J. C. & Anderson, W. F. Germ-line gene therapy: a new stage of debate. Law Med. Health Care 20, 26-39 (1992).

56. Schnicke, A. E. et al. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science* 278, 2130-2133 (1997).