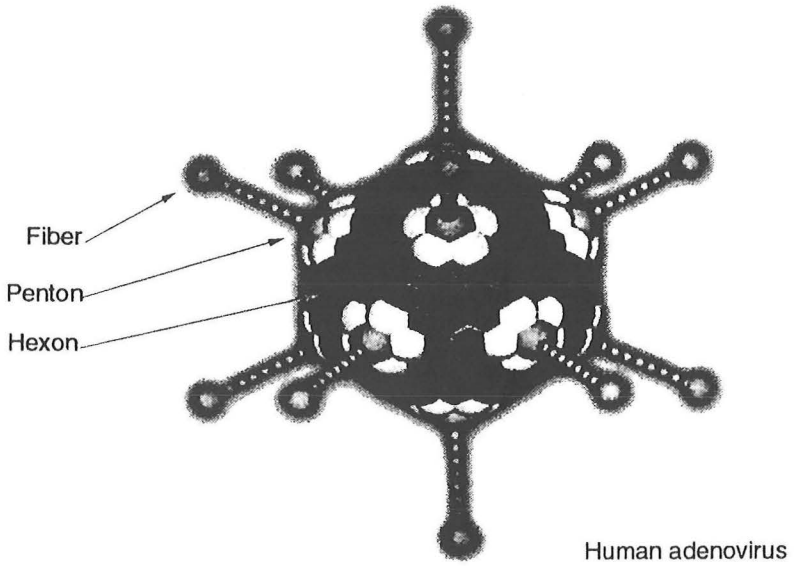
Gene Therapy 2000:

The End of the Beginning or the Beginning of the End?



Human adenovirus

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INTRODUCTION

The beginning of the last decade saw the first *attempt* at gene therapy, which is defined as the introduction of DNA into humans with therapeutic intent. As we begin this next decade, the first *successful* gene therapy has just been achieved. The purpose of this review is to highlight the progress of gene therapy in the last ten years, to see how this early success was achieved, and to draw lessons for the future.

GENE THERAPY 2000: The "race" is over. Now comes the hard part.

Correction of SCID-X

On April 28, 2000, the first convincingly successful gene therapy was reported in an article in Science (1). The key findings had been announced at the American Society of Hematology meeting in December in New Orleans. The finding was picked up by Reuters' news service but did not receive attention in the wider media. The facts are these: two unrelated boys with severe combined immunodeficiency (SCID, or "bubble boy" disease) have been free of disease for one year following removal of some of their $CD34^+$ hematopoietic stem cells, transduction of the cells *ex vivo* with a retrovirus containing a cDNA (which they lacked), and reinfusion of the infected cells three days later. In this lecture, I will provide the background needed to understand this pivotal experiment and assess the broader significance of these results.

The two patients had the most common form of human severe combined immunodeficiency, termed SCID-X1 (2, 3). SCID-X1 is an X-linked inherited disorder characterized by an early block in T and NK lymphocyte differentiation, due to an underlying deficiency in a subunit common to several cytokine receptors. This subunit, termed γc, is an integral part of the receptors for IL-2, -4, -7, -9, and -15 (Fig. 1). It is the most common severe combined immunodeficiency in man, accounting for about 50% of cases.

SCID-X1 is a lethal disorder that is curable by allogeneic bone marrow

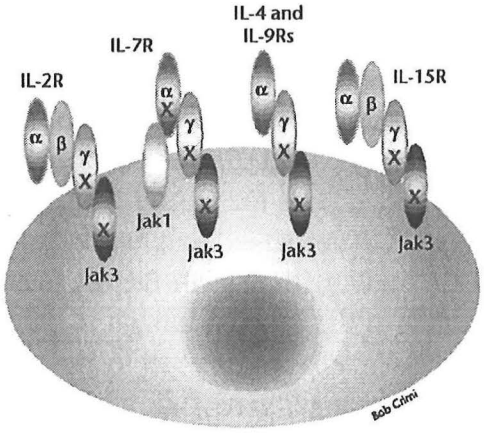

Fig. 1: Cytokine receptor defects known to cause SCID.
Mutations in the gene encoding the common gamma chain (γ) for all five receptors (Rs) result in SCID-X. Mutations in the gene for Janus kinase 3 (Jak3), which transduces the receptor signal from γ, cause autosomal recessive SCID that is phenotypically identical to SCID-X1. Finally, mutations in the gene encoding the chain of the IL-7 receptor also cause autosomal recessive SCID.
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transplantation. However, only a fraction of affected children have compatible bone marrow donors, and graft vs. host disease and defective B cell development remain a problem in the disease. Gene therapy seemed a particularly attractive option because the molecular lesion in SCID-X is well defined, and because the patients lack an immune system, so that immunological responses to the therapeutic protein or vector would presumably pose one less barrier to effective therapy. Another form of SCID (due to adenosine deaminase deficiency) was the target of the first gene therapy in 1990. In those few cases, efficacy of the gene therapy could not be assessed because the children received (and continue to receive) enzyme replacement therapy, which is an effective treatment for the disorder (4).

The attractiveness of this entity as a target for gene therapy was underscored by an experiment of nature, reported in 1996 in the NEJM (5) and studied more fully in a recent follow-up paper (6). This case involved a male boy of 12 months of age with well-documented SCID-X based on family history, clinical symptoms, and genetic evidence of a defect in the γc gene in B-cell lines derived from his blood. Interestingly, instead of the expected absence of peripheral T cells, he had nearly normal numbers of T cells in his blood. Furthermore, although he had been very ill as an infant, he had done well during his second year of life. Further investigation by his astute clinicians revealed that his normal T cells arose as a result of a reversion of his γc point mutation in a single cell. Normal T cell clones grew from this single cell, restored his immune system, and caused him to be clinically well for at least 5 years. It became clear that if a single cell could give rise to a diverse T-cell repertoire in this child, then successful transduction of γc into very few precursor cells in other children with SCID-X had a high likelihood of success. Potentially, only one cell might be needed to correct the disorder.

Much to their credit, these investigators took a systematic, yet expeditious, approach in moving toward their goal of supplying normal γc function to patients with SCID-X. Perhaps because they knew that long-term correction, but not a high efficiency of transfection was needed, they chose a well-studied and commonly used retrovirus for their studies. First, γc gene transfer was demonstrated in EBV-transformed SCID-X lymphoblastoid B cell lines, with successful reconstitution of IL-2 receptor expression and function (7-9). Next, in a particularly ingenious paper, γc was transduced into bone marrow cells from two SCID patients. The successful transduction of hematopoietic stem cells was demonstrated. In addition, the experimental system was cleverly manipulated to show that NK cells, which are also defective in SCID-X, require contributions from IL-7 and IL-15 for their development (10). In the next stage, purified $CD34^+$ (stem) cells collected from SCID-X patients were transduced with retrovirus and used to establish human-murine fetal thymic organ cultures (11). In these experiments, this unique system was used to establish the optimal conditions for transfection of $CD34^+$ cells to promote T cell proliferation and differentiation. They found that two factors were particularly important: prestimulation of the $CD34^+$ cells with growth factors (stem cell factor (SCF), Flt3 ligand, and IL-3), and transduction of the cells on human recombinant fibronectin-coated plates. They found that $CD34^+$ cells were able to mature into $CD4^+/CD8^+$ and $CD4^+$ cells with T cell receptor rearrangements following retroviral

transfer of γc. This showed that restoration of the γc/JAK/STAT signaling pathway during the early stages of thymocyte development could correct the T-cell differentiation block in SCID-X1 stem cells. Finally, they (12) and others (13) used a murine retrovirus to correct immunodeficiency in a γc-deficient SCID mouse model, using a similar *ex vivo* approach. It should be noted that the mouse model does not completely recapitulate human SCID. For instance, in mice, B cell numbers are markedly decreased, whereas in humans, the numbers are normal but the B cells are functionally abnormal. In addition, some abnormal activated T cells accumulate in SCID-X mice, leading to inflammatory bowel disease and splenomegaly, which are not features of the human disorder (12). Despite these differences, the gene correction restored normal B and T cell development, including normal T cell mitogenic responses, B-cell responses, production of antibodies, and restoration of gut-associated lymphoid tissue. These changes were stable in the mice for at least one year (to the time of publication).

In parallel work, normal dogs were treated similarly using an *ex vivo* approach (14). Interestingly, these dogs had intact immune systems. In these experiments, peripheral blood cells expressing the human γc could be readily detected, but disappeared in the animals between 19 and 34 weeks. Three dogs were treated with low doses of cyclosporine and prednisone, whereupon the γc expression reappeared and became stable up to 16 months (the time of longest follow up). γc-expressing cells comprised 25% of the dogs' lymphocytes.

The first successful human study followed closely on the heels of the preclinical work (1). The study reported on two patients, aged 11 months and 8 months, with SCID-X. One patient had a mutation causing a receptor lacking the cytoplasmic tail and the other had a protein truncated before the transmembrane domain that was not expressed at the cell surface. Marrow was harvested from the children under anesthesia and $CD34^+$ cells were collected by methods that are now routine; 10×10^6/kg from one patient and 5×10^6/kg from the other. The cells were transferred to special fibronectin-coated containers and cultured in the presence of stem cell factor, PEG-megakaryocyte differentiating factor, IL-3 and Flt-3 ligand. Retrovirus supernatant containing 5×10^5 infectious particles/ml was added. (This is a low titer when compared with other vectors in common use, such as adenovirus). The viral supernatant was added daily for each of three days, then the cells were centrifuged, washed twice, and infused back into the patients. At the time of infusion, about 35% of the cells were expressing the transgene. As shown in Fig. 2, T-cells ($CD3^+$ cells) began to appear in the peripheral blood by day 30. Surprisingly, expression of the γc transgene was equivalent to wild-type expression, and the resultant T cell population was shown to be polyclonal and diverse. In addition, antibodies to tetanus and diphtheria toxoids were found (after immunization) in the sera of both patients, along with nearly normal levels of IgG and IgM. One patient produced IgA and the other did not. γc-expressing NK cells returned by day 30 in one patient and day 150 in the other. Both patients left protective isolation at three months and were clinically well at one year. Two further patients have also been treated and are reported to be doing well. Although one year is preliminary, the optimistic view, guided by the

example of the patient with a spontaneous reversion (5), is that the transgene expression will be long-lived.

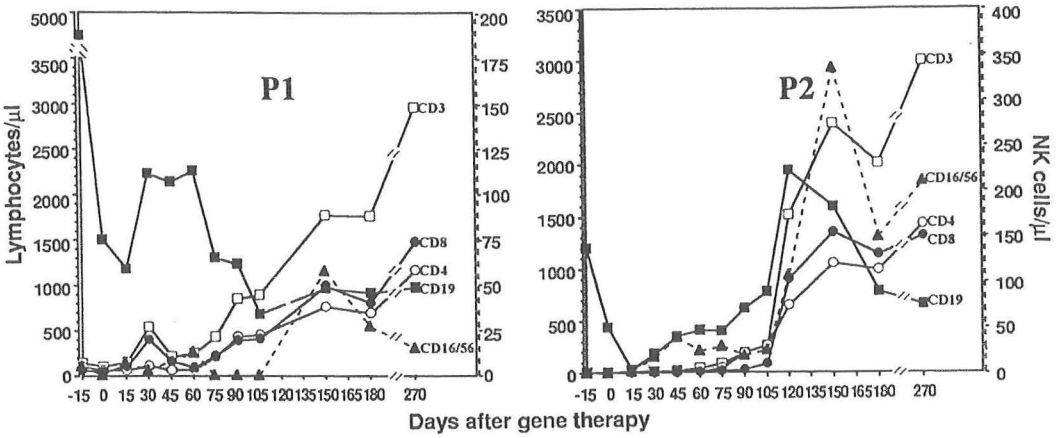

Fig. 2. Longitudinal study of lymphocyte subsets from patient 1 (P1) and patient 2 (P2). Absolute counts of T cells ($CD3^+$, $CD8^+$, and $CD4^+$), B cells ($CD19^+$), and NK cells ($CD16^+$, $CD56^+$) are shown as a function of time. From reference (1).

This case illustrates that curative gene therapy is possible, and that a logical, careful, systematic approach was the key to progress. However, this was the best-case scenario--it took advantage of the strong positive selective pressure imparted by the growth factor receptor on the recipient cells, and was used to treat patients without functional immune systems. However, at least one other report of clinical benefit from gene transfer has appeared--the use of AAV-factor IX to treat hemophilia B.

Treatment of Hemophilia B

Hemophilia B is an X-linked disorder caused by a very large number of mutations in the factor IX gene. This severe bleeding disorder is 10 to 20 percent as common as hemophilia A, with a prevalence of 20 per million (15). The residual activity of factor IX in hemophilia B varies according to the underlying mutation. About 80% of patients have severe disease (<1% residual activity). Interestingly, in contrast to hemophilia A, antibodies to factor IX develop in less than 1% of treated patients. Only patients with large partial or whole gene deletions seem to be at risk--the risk is about 50% among these patients and less than 1% for all others (16, 17). These findings are consistent with recent understanding of antigen processing in the ER (18), which predicts that small amounts of newly-synthesized albeit unstable mutant proteins will be degraded in the ER and effectively tolerize the host.

Patients with hemophilia B suffer from spontaneous bleeding into joints, muscles, and the brain, delayed but prolonged bleeding from minor cuts, and severe bleeding from lacerations. Even minor surgical procedures are undertaken with trepidation (and factor coverage). Before the availability of factor replacement, crippling joint contractures were

a major problem. Treatment consists of self-administration of factor IX, which has all but eliminated joint disease; however, internal bleeding and intracranial hemorrhage remains significance sources of morbidity and mortality. Hemophilia B was chosen as an early target for gene therapy because it is fairly common (though not nearly so at hemophilia A) and because factor IX is a small protein that undergoes fairly simple processing. (This is in contrast to factor VIII, which is encoded by a very large cDNA and undergoes very complex processing in endothelial cells).

It is pertinent here to consider the molecular biology of human factor IX, including what is known about its biosynthesis and processing. Human factor IX is a single polypeptide chain of 415 amino acids and has a molecular weight of about 55,000, about 20% of which is carbohydrate (19). The protein has four domains, a γ-carboxyglutamic acid domain, a growth factor domain, an activation peptide domain and a catalytic domain. Like Factor VIII, it participates in the intermediate phase of the intrinsic pathway of blood coagulation. It circulates in plasma as an inactive zymogen that is activated by proteolytic cleavage by factor XIa or by a complex of factor VIIa and tissue factor. Activated factor IXa then participates in a complex with activated factor VIII in the presence of a phospholipid surface and calcium ions to activate factor X. Since factor IX and factor VIII form a complex to perform a common function, mutations in either factor produce a similar clinical picture. Thus, hemophilia A and hemophilia B are clinically indistinguishable.

Factor IX is synthesized primarily in the liver. A signal peptide is removed cotranslationally and a further 18 amino acid propeptide is removed in the ER to generate the mature peptide present in plasma. The propeptide functions as a recognition site for the Vitamin K-dependent carboxylase, which catalyzes the formation of glutamic acid residues to gla residues. When infused into patients with hemophilia B, there is an initial clearance with a half-life of 50 min, followed by a slower disappearance with a half-life of 24 to 30 hours. It has been estimated that 120 to 180 μg/kg turns over daily (19). This corresponds to a daily production of 7 - 10 mg of factor IX per day for a 60-kg man. Since pure factor IX has a specific activity of about 500 U/mg, which corresponds to 3500 to 5000 units per day.

Preclinical studies of Factor IX gene therapy have been successful. In 1997, an AAV-human Factor IX was used in normal and immunodeficient mice (20). In the normal mice treated at a dose of 2 X 10^{11} vector genomes/animal, transgene expression was seen but no plasma level of human factor IX could be detected because of neutralizing antibodies. In the immunodeficient (RAG^-/RAG^-) mouse, therapeutic levels of factor IX were maintained for over 12 months (Fig. 3). No inflammation was noted at the site of injection (as had been seen for adenoviral vectors). This construct used a very strong CMV promoter/enhancer and an intron to boost expression. When DNA from the injection sites were analyzed 3 months later, it was found to have incorporated randomly and to have formed head-to-tail tandem repeats of two genomes per insertion site. Correction of hemophilia B has been reported in a dog model using AAV vector containing canine factor IX sequences injected into the portal circulation (21). In this study, it was shown that use of canine rather than human sequences was important. The

stable expression of therapeutic levels of factor IX (1.4%) in three dogs for over 2.5 years, with a reduction in bleeding episodes from 5 times per dog per year to 1 episode in one dog over the next year.

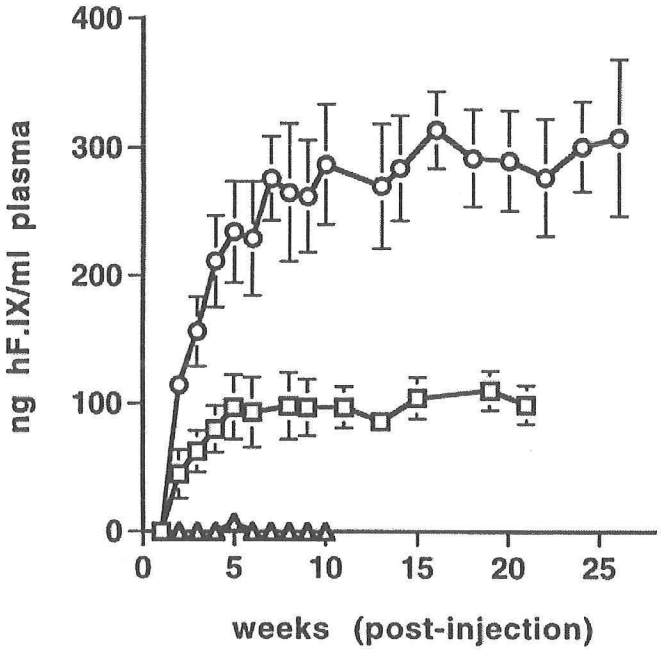

Fig. 3. Plasma concentration of hF.IX in experimental mice as a function of time after intramuscular injection with AAV-hF.IX. Triangles, C57BL/6 mice after i.m. injection of 2 X 10^{11} vector genomes/animal. Squares, Rag 1 -/- mice after i.m. injection of 1 X 10^{10} vector genomes/animal. Circles, Rag 1 -/- mice after i.m. injection of 2 X 10^{11} vector genomes/animal. From reference (20).

In the human study, three patients with hemophilia B were injected intramuscularly with a recombinant AAV vector containing human factor IX (22). The study has a dose escalation design, and these three patients received the lowest dose (2 X 10^{11} vector genomes (vg)/kg with 2 X 10^{12} vg/kg and 1 X 10^{13} vg/kg doses planned). All three patients had baseline levels of Factor IX of < 1%, and all had point mutations leading to amino acid changes that reduced (24% in one patient) or eliminated (<1% in two patients) circulating factor IX antigen. Two of the patients had very severe clinical courses and one had a milder course, requiring self-injections of factor IX around 4-5 times per year.

For injections, patients received recombinant factor IX coverage to 100%, and under ultrsound guidance, the vector was injected percutaneously into 10-12 sites in the vastus lateralis muscle of each anterior thigh. Each site received 0.25 to 0.5 ml and sites were at least 2 cm apart. There was evidence for biological effect as assessed by factor IX levels and activated partial thromboplastin time (PTT). Unfortunately, data from only two of the three patients was presented. The factor IX levels were not entirely convincing, as levels in this range are notoriously difficult to measure. This was commented upon in the paper and three independent laboratories were used to make these measurements. The PTT data might have been more compelling than the factor assays, except that factor IX infusions continued to be given; although 14 days were allowed to elapse before measurements, one cannot be sure that the corrected PTT were not the result of exogenous factor IX, because baseline PTT data 14 d after therapy was not

available in these patients. Patient 1 had a decrease in factor requirement of 50% and patient 2 of 80%. Results in patient 3 were not reported (Fig.4).

In this case, the efficacy of the treatment was not 100% convincing because of a lack of baseline data. It would have been desirable to include baseline PTTs in these patients, and the selective reporting of data was annoying. However, one can be optimistic about this study because the dosage level in 50 times lower than the dosage that was easily tolerated and therapeutic in the preclinical studies. Encouraging data in primates (unpublished) supports this optimistic view.

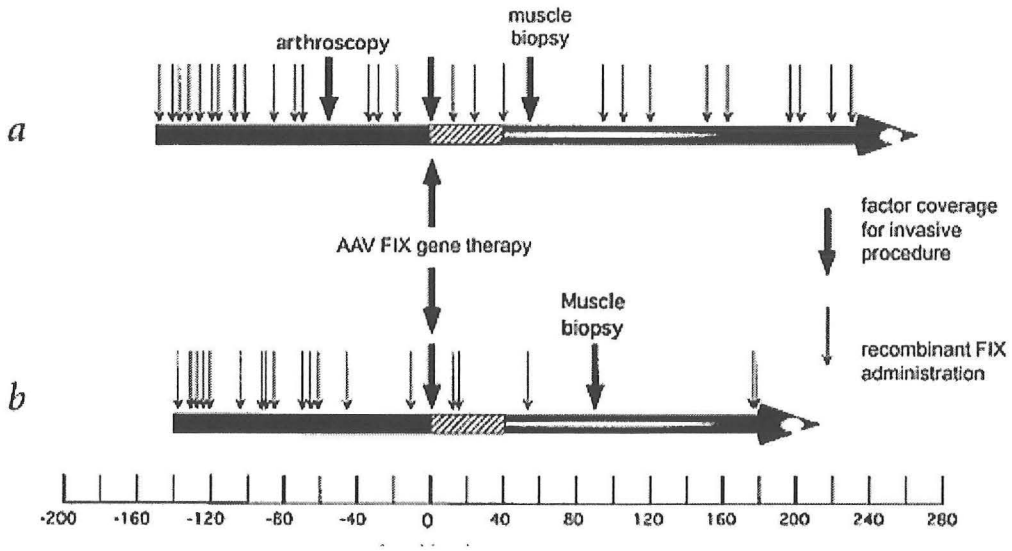

Fig. 4: Factor usage for patients A and B. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. The horizontal line denotes time; the scale at the bottom is marked in 20-day increments. Arrows denote infusion of F.IX concentrate for spontaneous bleeds (thin arrows) or invasive procedures (thick arrows). The thick vertical arrow in the middle of the chart denotes the date of vector infusion. The hatched bar on the timeline denotes the initial six-week period during which transgene expression is expected to be low based on animal studies. All patients have baseline F.IX levels <1%. From reference (22).

Angiogenic treatment of ischemic limbs

A third vignette concern the use of growth factor genes to promote neovascularization, in ischemic limbs or myocardium (reviewed in (25)). I present these data because they have been put forth as an example of "successful" gene therapy. However, in this case, while the results showing biological activity are clear, the clinical efficacy is less clear. I believe that the case illustrates an example where the appropriate target for gene therapy and the optimal therapy itself are not well defined.

The potential uses of angiogenic growth factors were anticipated by the work of Judah Folkman over 20 years ago (26). Preclinical studies have demonstrated that angiogenic growth factors can promote the development of collateral circulation in animal models of limb and myocardial ischemia (27, 28). These studies were first extended to human subjects using gene transfer of naked DNA encoding vascular endothelial growth factor (VEGF) for the treatment of critical limb ischemia (29). A dose escalation design was used, and at a dose of 1000 ug of plasmid DNA, 3 out of patients were shown to have improved blood flow and no rest pain. At a dose of 2000 ug, angiographic evidence of new blood vessel formation became readily apparent. Changing the mode of delivery from intraarterial to intramuscular reportedly improved the findings (30). Objective findings included improvement in the ankle brachial blood flow index, angiographic evidence of new collateral blood vessels, and MRI evidence of improved lower extremity blood flow (Fig. 5). Three out of seven patients treated were said to have been spared amputation. This was an uncontrolled study so we cannot firmly conclude that these anecdotal results were the effect of the treatment. A follow-up study on 90 of these patients similarly treated reported a high incidence of lower-extremity edema (30%) suggesting a biological effect. It was noted that the response to VEGF gene transfer was most robust in young patients with premature atherosclerosis due to Buerger's disease (thromboangiitis obliterans) (31), and explanations posited about why the effect may be greater in the young than in the old. It is hoped that with the collection of more data (including appropriate controls) we will learn whether or not there is a benefit to this novel form of treatment.

Reportedly successful application of gene transfer for the treatment of myocardial ischemia in humans was claimed in 1998. This study involved direct intramyocardial

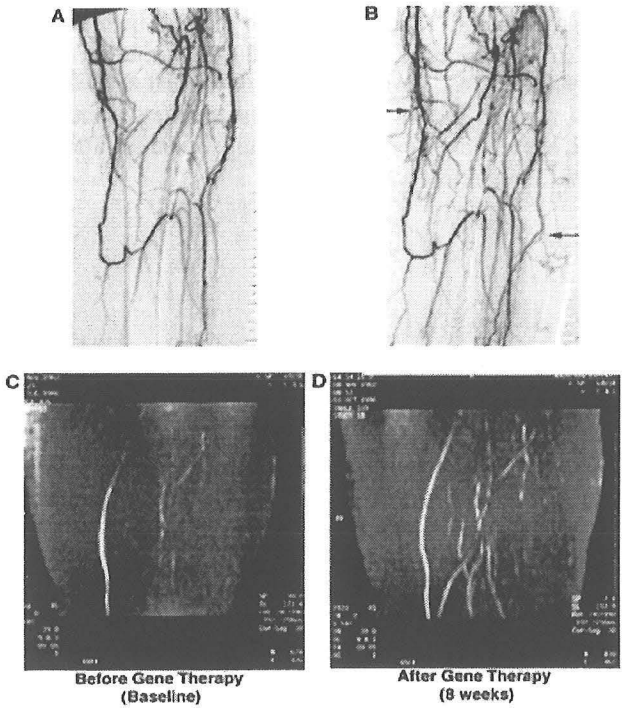

Fig. 5. A and B, Newly visible collateral vessels at calf level 8 weeks after phVEGF165 gene transfer. Luminal diameter of newly visible vessels ranged from 200 to >800 μm (arrow); most were closer to 200 μm, and these frequently appeared as a blush of innumerable collaterals. C and D, MRA before and 8 weeks after gene therapy. After gene therapy, signal enhancement is clearly evident, consistent with improved flow in the ischemic limb. From reference (30).

injection of phVEGF as sole therapy for myocardial ischemia refractory to conventional therapy (32). Although it was a phase I trial designed to study *safety*, the authors' major conclusion concerned *efficacy*. Five patients received four aliquots of 2 ml each containing a total of 125 ug of plasmid DNA encoding VEGF by direct injection through a mini-thoracotomy. All reported improvement. A placebo was not felt to be feasible and no control group was identified. Unfortunately, because of the limitations of the study, conclusions as to efficacy are really not possible. Of note, in a recent similar study in which growth factor was given intraarterially (33), a very strong and sustained placebo effect was seen. It is hoped that future trials will be better designed to address efficacy.

CURRENT GENE TRANSFER TECHNOLOGY

This section will review some of the methods developed over the past ten years for delivering DNA to tissues for therapeutic intent. This list is not all-inclusive, but serves as a sampling to illustrate some general principles and a range of approaches. Table 1 summarizes some of the key features of these DNA transfer vehicles, is shown below.

Table I. Advantages and disadvantages of gene-transfer vectors

Vector	Advantages	Disadvantages
Adenovirus	Very high transfection efficiency *ex vivo* and *in vivo* Transfects proliferating and non-proliferating cells Substantial clinical experience acquired Efficient retargeted transfection demonstrated	Repeat dosing ineffective owing to strong immune responses Insert-size limit of 7.5 kb Manufacture, storage, QC are moderately difficult Short duration of expression
Retrovirus	Fairly prolonged expression High transfection efficiency *ex vivo* Substantial clinical experience *ex vivo* Low immunogenicity	Low transfection efficiency *in vivo* Insert-size limit of 8 kb Transfects only proliferating cells Safety concern of insertional mutagenesis Manufacture, storage, QC are extremely difficult
Lentivirus	Transfects proliferating and non-proliferating cells Transfects haematopoietic stem cells	Safety concerns from immunodeficiency virus origins Manufacturing, storage, QC are extremely difficult Insert-size limit of 8 kb No clinical experience
AAV	Efficiently transfects a wide variety of cells *in vivo* Very prolonged expression *in vivo* Low immunogenicity	Insert-size limit of 4.5 kb Manufacture, QC are very difficult Little clinical experience Safety concern of insertional mutagenesis Repeat dosing affected by neutralizing antibody responses
Naked DNA	Manufacturing, storage, QC are simple and cheap Very low immunogenicity Clinical efficacy demonstrated in critical limb ischaemia Very good safety profile	Very short duration of expression in most tissues Very inefficient transfection *ex vivo* and *in vivo* Retargeting transfection very difficult
Cationic lipids	Relatively simple manufacturing, storage, QC Efficient transfection *ex vivo* Low immunogenicity Good safety profile	Inefficient transfection *in vivo* Very short duration of expression Little clinical experience Retargeting transfection difficult
Condensed DNA particles	Relatively simple manufacturing, storage, QC Efficient transfection *ex vivo* Low immunogenicity Good safety profile Retargeted transfection demonstrated	Inefficient transfection *in vivo* Very short duration of expression No clinical experience

From A. Mountain, TIBTECH 18:119-128, 2000.

Naked DNA

The major advantages of the use of naked (plasmid) DNA are its ease of manufacturing and storage, low immunogenicity, and safety profile. Its disadvantages include a very short duration of expression in most tissues, and very inefficient transfection *in vivo* and *ex vivo*. There is a great deal of current interest in naked plasmid DNA for vaccines, because of potential ease of manufacture and stability (34).

DNA Complexed with Cationic Lipids

DNA complexed with cationic lipids is one preferred method of transfection in the cell culture laboratory. It has the same limitations as naked DNA, especially with respect to *in vivo* efficiency. Recently, modifications to improve uptake, especially by the liver, have shown some promise. In general, clinical experience with this method is very limited.

Retroviruses and Retroviral Vectors

Retroviral life cycle. A great deal of excellent and readable information concerning the life cycle of retroviruses and their use as gene delivery vehicles can be found in a recent textbook (35). Some of this information will be briefly summarized here (36). Retroviruses comprise a large family of enveloped RNA viruses that are 80-100 nm in diameter, contain a 7-12 kb virion RNA that is linear, single-stranded, and of positive polarity. They replicate through reverse transcription of a virion RNA into a linear double-stranded DNA and subsequent integration of the DNA into the genome of the cell. In fact, the name "retrovirus" derives from the reverse flow of information, from RNA to DNA and back to RNA, which is in contrast to the "central dogma" of the flow of information from DNA, to RNA, to protein.

Retroviruses are classified as being either simple or complex. Simple retroviruses (Fig. 6) contain only three major protein coding regions, denoted *gag*, *pol*, and *env*. *Gag* (which stands for group specific antigen, not glycosaminoglycan) encodes internal proteins which form the matrix, capsid (external capsule) and nucleoprotein structures. *Pol* encodes reverse transcriptase and integrase enzymes, and *env* directs the synthesis of the components of the viral envelope protein. Gag, Pol and Env are polyproteins that are further processed by a protease, Pro, which is encoded after Gag in all simple retroviruses. In contrast to simple retroviruses, complex retroviruses contain information for additional regulatory proteins that do not appear in the viral particles and that arise from complex splicing events.

Seven different genera of retroviruses have been described--four simple and three complex. An example of a simple retrovirus is the Moloney murine leukemia virus, the most commonly used retroviral vector. Lentiviruses, of which HIV is a member, are examples of complex retroviruses. Retroviruses have provided invaluable insights into

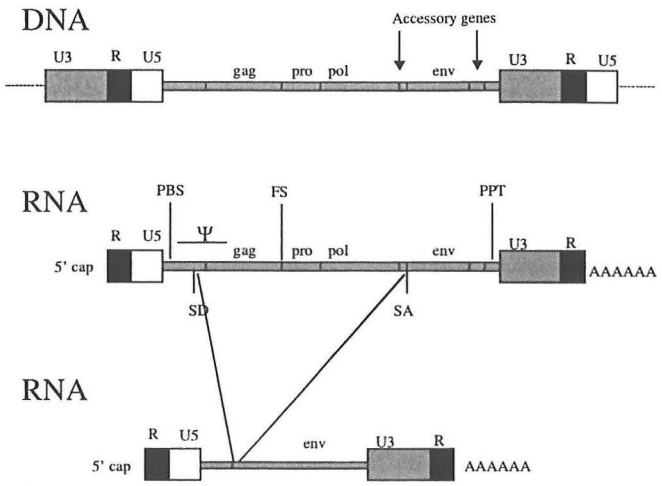

Fig. 6. Genetic organization of generalized provius. The provial DNA as it is inserted into host DNA is shown at the top, with the long terminal repeats (LTRs) composed of U3, R, and U5 elements at each end abutting cellular sequences. Sequences in the LTR that are important for transcription are marked. The *gag*, *pro*, *pol* and *env* sequences are located invariably in the positions shown in all retroviruses. The RNA that is the primary transciptional product is shown on the second line. Sequences that are important for replication and gene expression are shown in the approximate location in which they are typically found. (PBS) Primer-binding site; (ψ) encapsidation sequence; (SD) splice donor site; (FS) frameshift site; (SA) splice acceptor site; (PPT) polypurine tract; (PA) polyadenylation signal; (AAA) poly (A) tail. The spliced mRNA for the Env protein is shown on the third line. See reference (36) for more information.

oncogenesis, provided essential tools (reverse transcriptase) for biotechnology, and are widely used for expression of cloned genes in mammalian cells.

Viruses enter host cells through highly specific interactions of viral glycoproteins and cell membrane receptors, which leads to the fusion of viral and host cell membranes. The viral RNA enters the cytoplasm as a ribonucleoprotein particle that contains reverse transcriptase and a double-stranded viral DNA. The viral DNA is made through a fascinatingly complex process that involves the jumping of reverse transcriptase twice and duplication of sequences at the 5 and 3' ends of the virion RNA, the long terminal repeats (LTRs). The LTRs contain the promoter elements that drive gene expression.

The viral DNA is translocated into the nucleus where the linear copy of the retroviral genome is inserted into the chromosomal DNA in a process catalyzed by the viral integrase. While integration is not completely random, the potential number of integration sites is enormous and they are widely dispersed throughout the host genome. The viral sequences, LTR-gag-pol-env-LTR, are now referred to as a provirus, which now may be regarded as a cellular gene, with control of proviral transcription remaining largely with the LTR. (In complex viruses, the accessory proteins play an active role in controlling the level of transcription and relative amounts of different gene products). The viral mRNA is transcribed by cellular machinery, and the translated polyproteins and progeny RNA are assembled near the plasma membrane into viral particles that bud from the plasma membrane, often without any disturbance of the cell. Cleavage of the polyproteins occurs within the newly formed viral particles and is carried out by protease with the help of cellular proteases.

Therefore, one of the most important features of retroviruses is the ability to integrate and become a permanent part of the cell.

How and why do retroviruses take over cellular genes to become oncoviruses? It turns out that retroviral particles contain two copies of their genome linked by regions at the 5' end, so that they are in fact diploid. Consequently, when two genetically distinct retroviruses infect the same cell, stable genetic recombinants may be formed. This heterozygosity and facile recombination probably facilitates the inadvertent incorporation of cellular genes in place of viral genes, the replacement of which are tolerated because of the diploid state. Transduction of cellular genes has only been observed for simple retroviruses but not complex retroviruses, for reasons that are incompletely understood. Acutely transforming retroviruses transduce cells by virtue of the presence of the cellular oncogene. Retroviruses that have not transduced a cellular oncogene can nonetheless induce tumors in animals through inappropriate expression of a cellular gene under the control of a nearby viral LTR. Other viruses use less common mechanisms, such as the use of a viral encoded regulatory protein to change expression levels of cellular genes.

Almost all acutely transforming oncogenic retroviruses fail to replicate or produce infectious particles. However, they can be made to do so by superinfection with a helper virus. This principal led to the discovery of simple endogenous retroviruses, first in fowl and mice, but also in humans.

Retroviral vectors. Retroviruses are considered good gene delivery vehicles because of their ability to stably integrate into the host genome (reviewed in (37)). In addition, the presence of cellular oncogenes in transforming retroviruses demonstrated that portions of the viral genome may be replaced with foreign DNA without affecting viral entry or integration. Even though these changes resulted in defective replication, this is considered to be desirable because of safety concerns. The defect in replication was overcome, early on, by the use of helper virus, and later, through the development of packaging lines that would provide all of the retroviral proteins in *trans* but not produce any replication competent virus. The process of gene transfer and expression through the use of replication-defective virus is referred to as transduction rather than infection to emphasis this point. The newest retroviral vectors have been improved by removing all viral coding regions and by keeping other viral genes to a minimum. Another improvement has been the extension of the host range of retroviral vectors by using the VSV surface protein in place of Env (pseudotyping). This also allowed for improved ability to prepare concentrated viral stocks, because VSV is more stably associated with the virions. Additional improvements have included the use of multiple promoters, insertion of genes in the reverse orientation, and the use of internal ribosome entry sites (IRESs).

The minimum requirements for retroviral vectors are shown in Fig. 7. Important cis-acting elements include:

1. a promoter and polyadenylation signal
2. a viral packaging signal (psi) to direct incorporation of vector RNA into virions
3. signals required for reverse transcription, including a binding site for a primer (cellular tRNA) at the RNA binding site, PBS
4. A polypurine tract (PPT) for initiation of first and second-strand DNA synthesis
5. A repeat (R) region at both ends of the viral RNA that allows for reverse transcriptase to jump between templates in making two strands
6. Short partially inverted repeats at the termini of the viral LTRs required for integration.

Note that introns contained within the insert will be removed during vector replication, unless sequences to protect them are included.

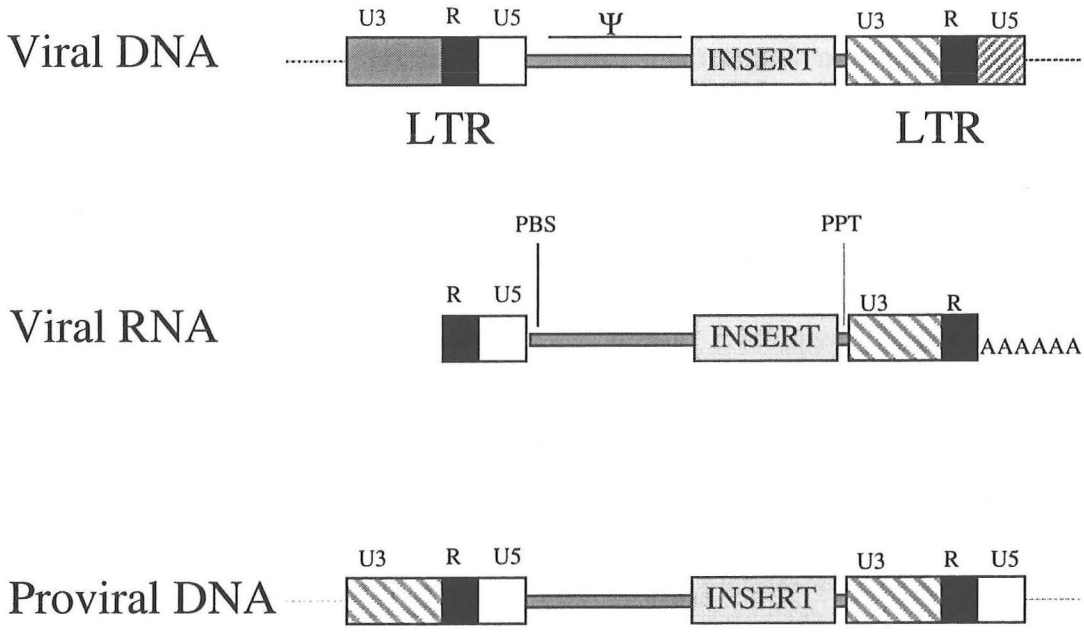

Fig. 7. Important cis-acting elements in retroviral vectors. (U3) unique 3' LTR RNA; (U5) unique 5' LTR RNA; (R) repeat sequence. For details, see reference (37).

Most retroviral vectors are designed to produce a protein of interest in one or more cell types. The simplest design uses the viral LTR to drive transcription of a cDNA encoding the protein. However, many adjustments to this simple design are possible--some permutations include the use of an internal promoter to drive transcription, or insertion of selectable markers. Two or more proteins may also be expressed, and several different strategies are available. With each permutation, difficulties can arise, not only with level of expression but with the level of viral production. Self-inactivating vectors are designed to prevent reactivation of a latent provirus, through deletion of the enhancers or promoters in the U3 region of the 3' LTR. These changes are copied into the 5' end of the LTR, inactivating transcription from the LTR after one round of transduction, but still allowing transcription from internal promoters. The earliest self-inactivating vectors could only be produced at low titer and had a tendency to produce viruses with intact LTRs by recombination. Newer strategies appear to be more promising. Improvements in vector design in terms of viral production and transduction efficiency have also been made.

Problems and limitations. Some cDNAs have coding regions containing sequences that, for unknown reasons, inhibit viral RNA accumulation. Factor VIII has such a region that reduces vector titer about 100-fold (38). There is no lower limit on cDNA insert size. The upper limit depends upon the retrovirus used, but 8 kb is typical. Most cDNAs can be accommodated within this constraint. One of the biggest limitations of simple retroviruses is said to be the inability to transduce non-dividing cells. This limitation appears to result from the inability of the virus to gain entry to the nucleus; nuclear membrane breakdown during cell division appears to be required. Lentiviral vectors, however, seem to have signals for nuclear import and can readily infect non-dividing cells. Suppression of viral gene expression has been reported, but is inconsistently observed, and still remains a mysterious phenomenon (39).

Another difficulty in the use of retroviruses is the complex manner in which they must be prepared. Unfortunately, packaging of retroviruses has only been achieved in intact cells. Earlier packaging schemes used viruses with deleted regions that provide all the protein required for viral replication but which cannot replicate. Spontaneous generation of replication competent virus through recombination was a serious problem. Additional changes in the helper virus were added so that up to three recombination events would be required to generate replication-competent virus. Assays for the sensitive detection of helper virus in viral stocks have been developed. One of these is called the S+L- assay, which is a marker rescue assay based on a defective oncogenic retrovirus (40). The assay is based on the rescue of the defective oncogenic virus by replication competent helper virus to produce transformed foci on indicator cells. Antibodies to viral proteins in the host animal (or human) though not specific, are also routinely monitored to assess for the presence of helper virus.

Complement in human serum can directly lyse virions or can damage them in the presence of anti-O-galactosyl antibodies present in human serum. This is a problem with

virus produced from mouse packaging lines; fortunately, human packaging cell lines are now available.

The host range of retroviruses may also be a consideration in the design of retroviral gene therapy. Because the retroviral vector will now contain only the cDNA of interest and a few regulatory sequences, the host range of the virus is now a function of cDNAs engineered into the packaging line. Retroviruses are broadly classified as being ecotropic (infect mouse cells only) or amphotropic (both human and mouse). However, within these categories, a broad range of cell types can be transduced, albeit at differing efficiencies. Both cell lines and primary cells can be transduced. Attempts to engineer the tissue tropism of retroviruses have been made, largely by alterations to Env. Thus far, this approach has been limited by poor viral titers with the exception on the replacement of Env by VSV-G protein, which has produced vectors with broad host range and improved viral stability.

In summary, the most significant features of retroviruses are their relatively high efficiency of gene transduction and their permanent integration to (often) provide long-term gene expression. Safety issues related to insertional mutagenesis remain a concern.

Lentiviral vectors

The basic genome organization for HIV-1 is that same as for simple retroviruses (reviewed in (41)). However, accessory genes play crucial roles in viral replication. For example, Tat activates the HIV LTR promoter so that viral RNA is produced efficiently, and Rev promotes the transport of viral RNA form the nucleus to cytoplasm.

In contrast to simple retroviruses, HIV can infect nondividing cells, although this observation has recently been questioned (42). Both integrase and matrix proteins contain signal sequences for nuclear localization, and Vpr appears to bind directly to the nuclear pore complex, allowing entry of viral DNA into the nondividing nucleus. The complexity of HIV has made the development of vectors and packaging cell lines more difficult as compared to retroviruses. Newer HIV vectors have been designed. For example, replacement of Env genes with VSV-G, a trick applied to other retroviruses to improve viral purification and yield, has turned out to be useful. Packaging lines have been developed. Transduction of retinal, hepatic, muscle cells, and hematopoietic stem cells has been demonstrated (reviewed in (41)).

Many of the concerns raised by simple retroviruses have been raised in HIV as well. In addition, generation of wild-type HIV, though reportedly not observed, is still possible with the use of helper virus. Insertional mutagenesis is a theoretical problem, but if not considered to have occurred in natural infections. A possible problem could occur if person treated with an HIV vector was to become infected with HIV. The transduced gene could spread throughout the individual and even infect others. The use of suicide genes in the vector has been brought up but not adequately addressed. Vectors based on feline immunodeficiency virus (FIV) have been developed and may more easily gain acceptance (43).

Adenovirus

Adenovirus (Fig. 8) is a double-stranded non-enveloped DNA virus with a genome size of 36 kb, ample room for additional DNA (reviewed in (44)). It does not ordinarily integrate its genome into the host chromosome, so gene transfer is temporary. Adenoviruses can infect dividing or non-dividing cells and they cause colds in humans. The life cycle of adenoviruses is much simpler than that of retroviruses. The virus attaches to the cell surface by binding to a cell membrane protein, called the coxsackie virus and adenovirus receptor (CAR) and with a cell surface integrin. The bound receptor is internalized into clathrin-coated pits that form endocytic vesicles. The lower pH of the endocytic vesicle releases the virion, which gains access to the cytoplasm by an unknown mechanism. The virus associates with microtubules and arrives at the nuclear pore complex, and the viral DNA enters the nucleus. The viral DNA forms a complex with histones from the host cell that resembles chromatin. Adenoviral DNA does not integrate into the host genome and is replicated as an episomal element.

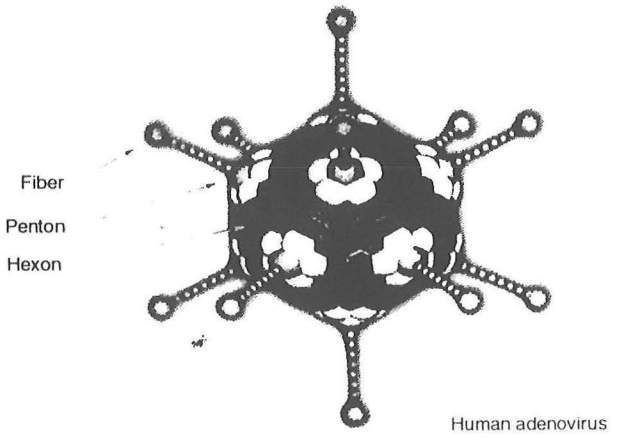

Fig. 8. Structure of human adenovirus, indicating the arrangement of capsid proteins. Riber and penton recognize cellular eceptors the mediate endocytosis of the virus. From reference (62).

The natural life cycle of adenovirus is divided into early and late phases. The early phase is defined as the synthesis of viral RNA from the viral DNA before the onset of viral DNA replication. Five viral regions, called E1A, E1B, and E2-4, are involved. E1A activates transcription and causes the host cell to enter S phase. E1B cooperates with E1A to induce cell growth. E2 contains three proteins, including a DNA polymerase, that are needed for DNA replication. E3 is a modulator of the immune response to viral infection, and E4 mediates the transition from early to late gene expression. There are five families of late mRNAs, designated L1-L5 that are involved in the production of capsid proteins.

All adenoviral proteins can be provided in *trans*; the only elements required for viral propagation and packaging are the inverted terminal repeats (ITRs) and a packaging signal sequence, called y. Therefore, over 25 kb of a transgene can be accommodated. Strong immune responses limited the use of early adenoviral vectors, in which only the E1 region (essential for viral replication) was deleted. "Gutless" adenoviral vectors have been developed that lack all but the two essential elements. As in the retroviral vectors, a "helper" virus must be used to package the transgene. Upon removal of the helper virus, the gutless vector can transduce cells and the expression can last for up to several months. However, low yield, poor stability and importantly, helper contamination are problems

that have yet to be overcome with the gutless vectors. The adenoviral vector that caused the death of the 18 year old with ornithine transcarbamylase (OTC) deficiency at Penn was a third generation vector that carried deletions of E1 and E4, but also contained gene sequences that have been associated with the development of immune responses (45).

Adenoviral vector titers of up to 10^{12} transducing units per ml can be achieved by transient transfection of host cell lines. Low levels of adenoviral proteins are produced from coding regions remaining in the vectors, triggering autoimmune response that kills infected cells and prevents further treatment with the vector. Because of the transient nature of the gene expression, and problems with strong immune responses, adenoviral vectors have been considered less desirable as compared to other viral vectors (37), but they remain useful research tools for cell culture and preclinical studies.

Adeno-associated virus

Adeno-associated virus (AAV) is a small, single stranded DNA virus that is a member of the parvovirus family (reviewed in (46)). It requires a helper virus, such as adenovirus, for replication. About 80% of the human population are seropositive for AAV. AAV is small and contains only two major genes, called rep and cap (Fig. 9). These genes are important for replication and encapsidation, respectively, and can be supplied in *trans*. AAV can integrate into the host genome. Wild-type AAV integrates into a single site on chromosome 19, but AAV vectors integrate randomly. The size limit for inserts is 4.5 kb. Effective titers are similar to retroviruses (10^7/ml).

AAV is the recent darling of the gene therapy world. Two important papers demonstrated long-term expression in muscle tissue after a single injection of the virus (47, 48). These papers showed that rAAV injection into muscle resulted in a prolonged expression of a marker protein or erythropoietin without toxicity to the animal and without eliciting a cellular immune response. Recent significant improvements in viral production, including adenovirus helper free production systems, have increased enthusiasm for AAV. Commercial viral production facilities now exist to meet the anticipated demand for AAV gene therapy vectors.

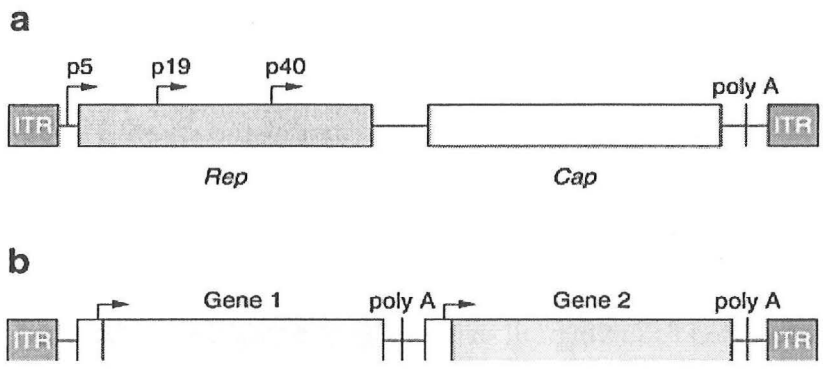

Fig. 9 (a) Structure of adeno-associated virus (AAV). The position of the rep and cap genes, the inverted terminal repeats (ITR), the viral promoters (p5, p19 and p40) and thepolyadenyl-ation site (poly A) are indicated. (b) AAV can be used as a vectorby inserting therapeutic genes between the ITRs. From reference (44).

A recent notable paper (49) compared the transduction of different cell types and regions in the mouse brain using different types of AAV, and demonstrated a remarkable ability of one type, AAV-5 to spread throughout the mouse CNS with a single injection, in contrast to other viruses, which stay localized. In addition, persistent expression of a transgene (over one year) was seen. This has generated a lot of interest in AAV for treatment of central nervous system disease. The approach to the central nervous system poses particularly difficult challenges, such as the necessity for treatment of post-mitotic cells, the heterogeneity of cell types, complex neuronal circuitry, and limited access (50).

ARISING TECHNOLOGIES

Several new technologies in preclinical development are particularly promising and deserve mention here.

Chimerasplasty

Several years ago, researchers discovered that oligonucleotides containing both DNA and RNA showed increased pairing efficiency with genomic DNA targets (51, 52), and this observation was exploited to achieve the correction of single nucleotide mutations in episomal and genomic DNA (53-55) in cultured cells. Chimeric RNA/DNA oligonucleotides have subsequently been designed and optimized for increased stability, resistance to nucleases, and improved localization to target sites in the genome. Fig. 10 shows a typical oligonucleotide used for such a gene correction experiment, which is sometimes referred to as chimeraplasty (in reference to the chimeric nature of the RNA/DNA hybrid). Features include: 1). Double-stranded regions that are capped by single-stranded thymidine hairpins; 2). The 3' and 5' ends are in juxtaposition but anchored by a GC clamp at the 3' end; 3). RNA residues are 2'-O-methylated to prevent degradation by RNase H; 4). The mismatch is part of a short DNA sequence flanked by longer stretches of RNA sequences. It appears that the RNA/DNA strand of the duplex is responsible for the initial pairing event, which is made more efficient by the presence of the RNA residues in the structure. The mismatch in the DNA portion of the homology strand activates the endogenous DNA repair process (56).

The chimeraplasty technique has been used recently in animals to create a factor IX deficient rat (57) and to partially correct hyperbilirubinemia in the Gunn rat model (58). The Gunn rat is a model of Crigler-Najjar syndrome type I (UDP-glucuronosyltransferase Type 1A1 deficiency). In both instances the chimeric RNA/DNA oligonucleotides were formulated with polyethylenimine (PEI) or encapsulated in anionic liposomes with modifications that enhance uptake through the hepatic asialoglycoprotein receptor. In the factor IX model, a 40% conversion of wild-type to mutant alleles was reported following tail vein administration, with a decrease in factor IX levels of 50% and a prolongation of the PTT. (Unfortunately, this better-than-expected response raises the possibility that the effect on coagulation was due to direct

liver toxicity, an issue that was not addressed). In the Gunn rat model, a clinically significant reduction in hyperbilirubinemia was observed in the rats (Fig. 11).

A major advantage of this technique is that normal, physiological and regulated levels of the target protein would be restored, and that silencing or loss of expression should not occur. This new technique would be terrific if it could be applied efficiently to hematopoietic stem cells for the correction of sickle cell anemia. Early results with hematopoietic stem cells has been disappointing (55), but did not involve some of the manipulations that were successful in the SCID-X case. I believe this technique holds enormous potential.

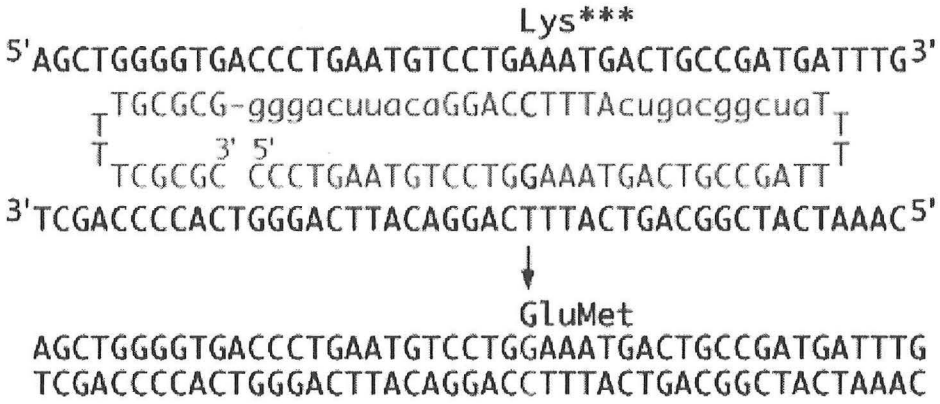

Fig. 10. Targeting strategy to correct the UGT1A1 frameshift mutation in the Gunn rat. The 2'-O-methylated RNA residues of the targeting RNA/DNA ON (gray) are indicated in lowercase and the DNA residues in capital letters. Blocks of 10 modified RNA residues flank both sides of a 9-residue stretch of DNA,which contains the base change required for correction. The ON sequence is complementary to 28 residues of genomic DNA spanning the site of mutation with the exception of a G base (bold) targeted for position 1206. The cell's endogenous DNA repair process mediates insertion of G at the target site, thereby correcting the frameshift mutation and restoring UGT1A1 activity. The folded double-hairpin structure containing four T residues in each loop, a 5-bp GCclamp, and the modified RNA residues significantly improve resistance to nuclease degradation. From reference (58).

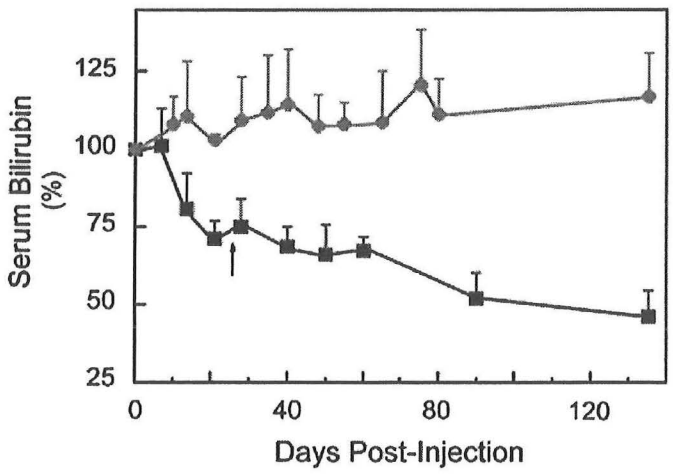

Fig. 11. Effect of UGT1A1 gene correction on serum bilirubin levels in Gunn rats. Animals were administered UGT1A1 (squares) or nonspecific (circles) ONs complexed to PEI or encapsulated in anionic liposomes. The dosage was repeated for all groups 30 days after the final injection of the first series (arrow). Each data point is the mean ± SD of 11 animals. From reference (58).

Human transposons

Another very recent interesting technology is the use of DNA transposons to insert gene-corrective material. These studies are in the preclinical phase but dramatic effects in hemophiliac mice have been reported (59). The technique utilizes two naked DNA plasmids that are co-administered and efficiently target the liver. One plasmid contains the corrective gene flanked by transposable DNA elements and the second plasmid encodes a transposase, adroitly named "Sleeping Beauty" transposase, derived from an ancestral Tc1-like fish transposable element (60). Transposase is an enzyme that binds to inverted repeats (on the plasmid) and catalyzes the insertion of the interposed DNA randomly into genomic DNA. When injected into immunocompetent hemophiliac mice, persistent expression (>5 months) of human factor IX was observed at levels of a few percent of normal, which was sufficient to shorten the bleeding time from >30 min to 4-7 minutes (nearly normal). Interestingly, readministration of the plasmids a few months later produced no immune response and approximately doubled the factor IX levels. As with the use of other integrating vectors, concerns about the potential for insertional mutagenesis must be addressed.

Systems for inducible or regulated gene expression

For the foreseeable future, unregulated production of therapeutic expression products is not an issue because levels of expression have been low and the therapeutic proteins chosen have had large therapeutic windows. In the future, the ability to precisely regulate the products of a therapeutic gene may become important as applications expand. For example, it will soon be possible to produce erythropoietin *in vivo* through the use of a transgene. Unregulated production of erythropoietin would have undesirable side effects. Much progress has been made in the area of systems in which expression is controlled by orally active drugs.

Four different systems have been explored in great detail, both *in vitro* and *in vivo* (reviewed in (61)).

1. tetracycline (Tet)
2. ecdysone and analogs
3. the progesterone antagonist RU486
4. chemical dimerizing agents such as rapamycin and analogs

The Tet system has become well established as a research tool. This system relies on the natural tet-controlled DNA binding domain of the E. coli Tet repressor fused to a heterologous transcriptional activation domain, usually herpes virus VP16. The transcription of genes with a minimal promoter and upstream TetR binding sequences can then be controlled by Tet (or doxycycline). Both positive and negative regulation (so-called "Tet-on" or "Tet-off" systems) have been described. Similar principles underlie the

ecdysone and RU486 systems. The major disadvantage of ecdysone is that little is known about the effects of ecdysone, an insect hormone, in humans. The RU486 system also has some drawbacks, such as undesirable kinetics. The fourth system relies on a different principle, which uses the drug rapamycin to bring a DNA binding domain and a transcriptional activator into close proximity. This system has been used to regulate epo expression in mice, but has not been successful in primates. Issues of immunogenicity of the heterologous proteins must be worked out before any of these systems will be useful clinically.

POTENTIAL USES OF GENE THERAPY: THE GOOD, THE BAD AND THE UGLY

As of May 25, 2000, a total of 425 gene therapy trials involving about 3500 human subjects are being conducted worldwide (Wiley Journal of Gene Medicine web site http://www.wiley.co.uk/genmed). Of these, 310 (70%) are U.S. trials, with most of the remainder being conducted in Europe. The diseases addressed by the trials include (Fig. 12): cancer (66%), monogenic diseases (13%), HIV (8%), and other (including cardiovascular and neurological), less than 4%. Another 10% are designated "gene marking" studies; virtually all of these are directed at cancer. I was surprised to learn that the gene therapy field is so dominated by cancer: Fully 66% of trials are directed at cancer patients, accounting for 70% of all patients enrolled. In fact, 37% of all gene therapy trials involve injecting a gene therapy vector directly into a tumor. A total of 2500 cancer patients are enrolled in gene therapy trials directed at cancer, as opposed to 66 patients in cardiovascular and neurological diseases combined. The types of genes being used in these studies is also informative: the majority are cytokines (16%), antigens, such as HLAB7 (15% of studies, but comprising 25% of all patients enrolled), suicide genes (12%), marker genes for cancer (9%), tumor suppressor genes (6%) and deficiency genes (12%), and receptors, 4%. Cytokines include GM-CSF, interferons, IL-12, Il-4, 6, 7, and combinations with IL-2. It is important to note that most gene therapy studies are quite small, generally less than 20 patients. 90% are in phase I or phase I/II trials, 9% have progressed to phase II and 2 studies are in phase III.

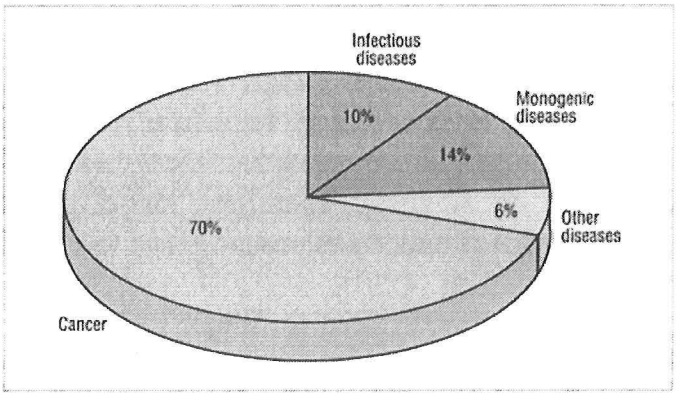

Fig. 12. Proportion of protocols for human gene therapy trials relating to various types of disease. Reproduced with permission from the Wiley Journal of Gene Medicine web site http://www.wiley.co.uk/genmed.

The "Good": Monogenic Disorders

I believe that it was no accident that the first successful gene therapy were directed toward monogenic disorders, because the underlying biology in most cases is very well understood. Current trials for monogenic disorders include α-antitrypsin deficiency, adenosine deaminase deficiency (SCID), Canavan disease (aspartoacylase deficiency), chronic granulomatous disease, cystic fibrosis, familial hypercholesterolemia, Fanconi's anemia, Gaucher's disease, hemophilia B, Hunter's syndrome, Hurler's syndrome, leukocyte adhesion deficiency, ornithine transcarbamylase deficiency, purine nucleoside phosphorylase deficiency (one form of SCID), and X-linked SCID. Particularly hopeful are disorders with defective but easily accessible stem cells, and those characterized by diffusible gene products. Lysosomal storage diseases, especially those that do not affect the central nervous system, are a particularly good next target, because hematopoietic stem cells are corrective and the proteins are, by and large, readily diffusible.

Monogenic disorders in which precisely regulated amounts of gene products are required (such as sickle cell anemia) will remain problematic, but newer techniques, such as chimeraplasty (if it could be made to work in hematopoietic cells) hold great promise.

The "Bad": Cancer Therapy

Because viruses have the ability to infect and kill cells, there has been interest in harnessing viruses to combat cancer for over 100 years. In the 1950's, adenoviruses were injected into solid tumors to assess their anti-tumor effects, which were found to be unimpressive (reviewed in (62)). However, because of safety and technical concerns, the viral vectors used in gene therapy have been inactivated to become replication-defective to remove any chance that they might spread and cause disease. But in preventing replication, the mechanisms that viruses use to grow and spread are also inactivated. The molecular weight of packaged viruses is well over one million, impeding diffusion and impeding passage through the small intercellular spaces. Furthermore, ligands on the surfaces of the cells may bind the virus and prevent further spread. All of these considerations limit the utility of viruses as efficient gene delivery vehicles. On top of this, a low efficiency of transduction further limits this approach. Oncologists are indoctrinated to believe (by experience) that effective tumor therapy (such as currently exists) requires reductions in cell numbers often expressed as a logarithm to the base 10 ("log kill")--several logs of cell kill are believed to be necessary for efficacy. Because cancer cells can simply divide and repopulate a tumor, any method that is less than 99.9% efficient is unlikely to excite most medical oncologists. Therefore, low-efficiency methods that are aimed at supplying (for instance) tumor suppressor genes to cells in solid tumors are met with reasonable skepicism.

Several more complex schemes are being investigated for tumor treatment and deserve comment. One is the use of a prodrug-activating system in conjunction with "suicide" gene therapy (63). A familiar example is the use of retroviral vectors in

combination with thymidylate kinase (TK) and ganciclovir, which has shown some modest effect in vivo for the treatment of brain tumors . The original principle of this scheme is that retroviral vectors transduce only dividing cells; in the case of brain tumors, the only (important) dividing cell are tumor cells, as neurons are quiescent. The retrovirus is made to produce thymidylate kinase, which is necessary for the toxicity of ganciclovir in cells. In addition, although the transduction may be inefficient, the scheme relies on a "bystander" effect whereby neighboring cells will be killed by diffusion of either the enzyme or the toxic metabolite from the transduced cell. These had some modest early success. However, one must realize that in many cases, tumor cells divide more slowly than many normal cells (and are defective in apoptosis instead), and may vary in their ability to be transduced. The approach is very complex and relies on many assumptions such as specific targeting, efficient transduction, lack of spread of toxic drug to neighboring healthy tissue, and therapeutic ratio of the drug for tumor vs. healthy tissue. Many of these issues have yet to be addressed before success could be considered likely.

Another extremely popular area for study is immunomodulation for the treatment of cancer (reviewed in (64)). A number of pre-clinical studies have shown that tumor cells engineered to secrete cytokines are rejected from a syngeneic host, in part by augmenting the host responses to the transplanted tumor. No fewer than 11 cytokines have been observed to produce this effect (IL-1, -2, -4, -6, -7, -12, -18, TNF-α, G-CSF, GM-CSF, or IFN-γ). Translation of these findings into the clinical arena has been slow. For example, isolation of primary autologous tumor cells that stably express high levels of the therapeutic cytokine has been technically difficult and poorly reproducible. More significantly, any therapeutic efficacy of the vaccines is lost after the first few days after implantation of the tumor cells; therefore, the mouse model poorly mimics the clinical situation in which established tumors are treated. Interest has shifted to the treatment of micrometastasis, and whether this approach will be fruitful remains to be seen.

The potent biological effects of viruses largely depend upon their ability to replicate within cells because great amplification of the original inoculum is possible. Safety concerns have limited cancer gene therapies to non-replicating viruses, but this limitation is being revisited. Given the relatively low efficiency of transduction of non-replicating viruses and the modest effects seen thus far (especially with respect to anti-tumor treatment), the use of replicating viruses is again being explored (65). The approach that has received the most attention is the use of a "selectively" replicating adenovirus (62); that is, the use of adenoviruses that may only replicate in tumor cells. Adenovirus replicates only in host cells in which a crucial cell checkpoint, p53, has been turned off, and it has a gene, E1B-55KkD, whose function is to bind and inhibit p53 so that the infected cell does not kill itself. If p53 were active, the infected cell would commit suicide and the infection would be limited. A majority of tumor cells are defective in p53 function, so the E1B-55KkD-deleted adenovirus should selectively replicate in only those cells, and the infection should not spread to normal healthy cells. That is the theoretical basis for ONYX-015 (now known as CI1042) which has been tested in phase I and phase II trials in advanced head and neck, pancreatic, ovarian,

colorectal and non-small cell lung cancer (by intratumoral injection). Clear evidence of a biological effect (pathologically demonstrated tumor necrosis) is seen in head and neck and colorectal cancers; however, the effect may be similar to that seen in studies done in the 1950s in which wild-type adenovirus was injected into tumors. Classically defined objective tumor responses with ONYX-015 have not been observed. It may be that the brisk immune response to adenovirus (which limits natural infection--after all, we don't all drop dead from colds) has limited the approach. The immune response includes not only humoral but also cytotoxic T cell responses and the release of cytokines. Deletion of genes important for these host responses may circumvent this problem, but then toxicity for the host and safety issues become a real concern. In addition, establishing initial infection may be difficult because tumors will be very heterogeneous with respect to cell surface receptors needed for viral entry. Of note, a large phase III trial (200 patients) combining ONYX-015 with conventional chemotherapy (5-FU and cis-platin) is scheduled to begin later this year.

Other selectively replicating viruses such as herpes simplex (66), vaccinia (67), and reovirus (68) are under development. Even genetically engineered bacteria, such as salmonella (69), are under investigation as anti-cancer vectors.

In summary, I believe that the large number of gene therapy trials directed at cancer reflects more the prevalence and importance of the disease rather than solid scientific opportunity or the likelihood of imminent success in this area. However, with the great strides in understanding of the molecular basis of cancer coupled with the recent availability of transgenic mouse models that better reflect human cancer, some important developments can be expected in the next decade, especially in preclinical models.

The Ugly

These include studies that address diseases for which effective therapy already exists or is imminent, or studies in which the target is really not well understood, or a combination of both. One or two examples may be reviewed in the lecture.

CONCLUSION

Ten years following the first clinical trial, successful gene therapy is a reality. From now on, gene therapy must no longer be considered a race to the finish but a healthy but emerging industry. I believe that in the next ten years, despite the recent high-profile tragedy (45), curative therapy for a handful of monogenic diseases will be well established. A few of us in the audience will refer patients for curative gene therapy; for instance, I consider it somewhat likely that curative therapy for sickle cell anemia will be available toward the end of the next decade.

What can clinicians and clinician-scientists do to contribute to this emerging field? I think the answer lies in developing a detailed understanding of disease pathology, so that appropriate targets are understood, not only for gene therapy but also for the development of other therapeutic modalities. For diseases in which long-term

correction will be needed, a deeper understanding of stem cell biology will be needed. The development of animal models that more closely resemble their human counterparts is needed. The recent development of mouse models that spontaneously develop tumors due to germ-line mutations should be exploited more fully before a large investment is made in human trials in cancer research.

More than ever, clinician-scientists involved in genetic research may come to rely increasingly on collaborations with biotechnology firms that will produce the clinical grade materials needed for gene transfer protocols. This situation creates new dangers and challenges. Academic researchers may feel conflicted between the desire to produce new basic fundamental insights into disease vs. the need to contribute to technological advances where the intermediate steps may have little value. This is the dilemma of the "translational" researcher. In the worst case, the translational scientist, who is not focused on basic biology, produces no basic insights and, lacking sufficient resources in an academic setting, is unsuccessful in developing new technology. The most successful clinician scientists will either play a role in the basic science underpinning the technology (virologists, "DNA" chemists) or will exploit familiarity with the new technology to gain fundamental insights into basic biological problems. Fruitful areas of study for future translational clinician scientists may include host-vector and host-tumor interactions, stem cell biology, the basic biology of the fate of DNA inside the cell, and mechanisms of DNA repair. The next ten years should see developments not only in the successful application of gene therapy but will also offer as-yet-unimagined avenues for interesting research.

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