Strategies in gene therapy

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Introduction

The enormous progress within the last 20 years in molecular biology and biotechnology has opened up new possibilities for the treatment of many diseases. A new discipline in medical sciences is evolving: molecular medicine. Investigations of the molecular basis of disease have led to fundamentally new insights with enormous consequences for treatment. The nature of the genetic defects underlying a large number of genetic diseases has been characterized and the biological consequences of the aberrant gene function have been described. Investigations of the type conducted by the Human Genome Project will continue to provide information about the genetic basis of complex diseases and will be essential to the identification of the considerable number of unknown genes responsible for the development of diseases of complex genetic and non-genetic etiologies. Pharmacology and human genetics are among the major disciplines which have been revolutionized by molecular medicine. The basis of this new discipline is the use of the methods of gene manipulation and gene transfer as therapeutic means.

Since gene transfer has succeeded in reversing disease-related dysfunctions in cell culture and animal models, application of new insights to human patients seems feasible [1]. In fact, the first clinical application of gene transfer was carried out in 1980 by Cline and coworkers; their aim was the treatment of β-thalassaemia [see 2]. At that time the whole concept and methods were too immature for success. The first clinical trial based on more advanced concepts, technology, and strategies was initiated in September 1990 for the treatment of a rare genetic disease, adenosine deaminase (ADA) deficiency, by Blaese and coworkers at the NIH in Bethesda [3] (see Fischer *et al.*; in this issue, pages 25-34). Since then, more than 500 patients have been treated worldwide on about 100 different protocols. Despite the fact that there is still only modest therapeutic success, these studies have paved the way for the development and application of yet more advanced strategies which will probably succeed within the next ten years.

Like most others in the field, I am not overly optimistic regarding the broad application of gene therapy in the near future. The technological limitations and the complex nature of the disease demand caution. Thus, successful treatment of the few «easy» diseases amenable to available technology must be demonstrated before accurate judgements about the future prospects of molecular medicine can be made. As we are at the beginning of gene therapy, it is the aim of this article to provide some essential background knowledge in order to enable the reader to estimate the prospects of this new technology for specific fields.

Candidate diseases for gene therapy

What makes a disease a potential target for gene therapy? First of all, every disease caused by a defect in a single gene is theoretically a suitable

target for treatment by gene transfer. In such a disease, the genetic defect would be corrected by transfer of the respective normal gene into the tissue(s) affected by the loss of gene function. However, the degree and extent of dysfunction vary greatly. A number of genetic diseases affect primarily only the function of one organ like the haematopoietic system or the liver (Table I). It is easy to imagine that gene transfer to one organ is feasible but treatment of the whole body or a variety of organs is more complicated. Two of the most important genetic diseases, Duchenne muscular dystrophy and cystic fibrosis (CF) find themselves somewhere in between these two extremes. In the first, all muscles are suffering from the absense of a functional dystrophin protein; local treatment might have some benefit but would not cure the disease. In CF most epithelia are influenced, to a varying degree by the lack of functional transmembrane chloride transport regulator (CFTR). However, the life-threatening effects are restricted to the lung and replacement of gene function in the lung would be the primary goal (see Coutelle; in this issue, pages 15-24).

Table I: Potential target diseases for gene therapy.

Disease	Deficient gene or target	Affected tissue(s)
Genetic diseases		
SCID	ADA	haematopoietic
Cystic fibrosis	CFTR	lung, intestine
Familial hypercholesterolaemia	LDL receptor	liver
Alpha-1-antitrypsin deficiency	A1AT	liver
Haemophilia A and B	factor VIII/IX	blood plasma
Duchenne muscular dystrophy	dystrophin	muscles
β-thalassaemia	β-globin	erythrocytes
Phenylketonuria	PAH	liver
OTC-deficiency	OTC	liver
Lysosomal storage diseases	various	liver, others
Cancer	various	one tissue
Non-genetic diseases		
Parkinson	dopamin synthesis	brain
Alzheimer	apo E/amyloid inhibition	brain
Arthritis	interleukin-1β receptor inhib.	joints
Hypertension	ACE inhibition ?	vessels
Restenosis	cell cycle inhibition	smooth muscular cells
AIDS	HIV gene inhibition	T-lymphocytes
Hepatitis (A-D)	viral gene inhibition	liver

Theoretically, every life-threatening non-genetic disease also could be a target for gene therapy provided the molecular nature of the disease is known [1]. A major group of diseases in this category is represented by the various forms of cancer. Cancer is indeed a gene based disease of a particular organ. However, the primary goal in this instance would be the selective elimination of tumour cells aided by special gene functions rather than the correction of a specific gene defect. Similarly, some severe chronic viral diseases like AIDS or the various forms of viral hepatitis also could be targeted. For these diseases, the aim would be inactivation of viral gene expression or gene function by the transfer of so-called antisense genes or anti-genes capable of counteracting target functions. The variant of this technology, downregulation of disease-disposing genes, potentially could also be applied to some forms of cancer and to a number of diseases that have not been characterized, e.g., hypertension and Alzheimer disease. In general, the potential applications of gene transfer technologies to the treatment of a wide range of disease appear to be very broad provided the technology is developed more adequately. Basically, a therapeutic gene will be just another drug.

However, some caution is advisable as to the unlimited application of this new concept. As long as reasonably good "traditional" treatments are available, there is no rush to develop gene therapy for a disease. As an example, phenylketonuria (PKU) should be mentioned. A well established nutritional strategy exists to prevent the severe manifestation of the disease. However, as soon as gene therapy for another genetic disease of the liver, like familial hypercholesterolaemia, will be well established on a routine basis, the same technology could also be applied to the treatment of PKU.

Gene delivery – general aspects

Gene therapy means transfer of a therapeutic gene into the target tissue and maintenance of the desired gene function for an acceptable time. Gene

delivery and maintenance or stability of gene function are the two major aspects of gene therapy which are under intensive investigation in many laboratories. The basic principle is rather simple. A gene coding for a specific function which has been isolated from a previously established library of genes is inserted into another DNA molecule (plasmid) which is able to multiply in bacteria. The resulting recombinant plasmid can be amplified in bacteria thereby generating large amounts of the gene. If the gene has its own expression-regulating elements (promoter), it can be introduced directly into mammalian cells, such as a patient's cells in culture (Fig.1).

A variety of simple means for gene transfer (transfection) in cell culture has been developed resulting in reasonable efficiencies measured by gene function one or two days after transfection (Table II). By that time, a considerable percentage of the transfected DNA has reached the cell nucleus and has been expressed by the cellular machinery. This phenomenon is called transient expression. The highest efficiencies of gene transfer (more than 50%) and transient expression can be achieved by direct microinjection of the DNA into the nucleus. Normally, only a small percentage of the DNA is able to integrate into the genome in a stable manner giving rise to genetically corrected cell clones. Integration of a normal gene where the corresponding defective gene resides occurs less frequently (0.1%). The mechanism responsible for the transfer is called homologous recombination. In cell culture, these stable genetic transformants can be isolated and expanded using selectable marker genes. These techniques, which were instrumental in basic research over the last 15 years, would be useful for gene therapy in cases where transplantation of genetically corrected cells is feasible. However, the expansion of cells in culture carries the risk of the accumulation of mutations and the occurrence of malignant changes. Therefore, two basic strate-

Table II: Basic methods for gene transfer in cell culture.

Method	Efficacy	Stability[a]
Physical method		
Electroporation	moderate	short/long
Microinjection	high	short/long
Particle bombardment	high	short
Chemical method		
Calcium phosphate	low/moderate	short/long
DEAE-dextran	low	short

[a] long stability is obtained by selection of clones with integrated genes

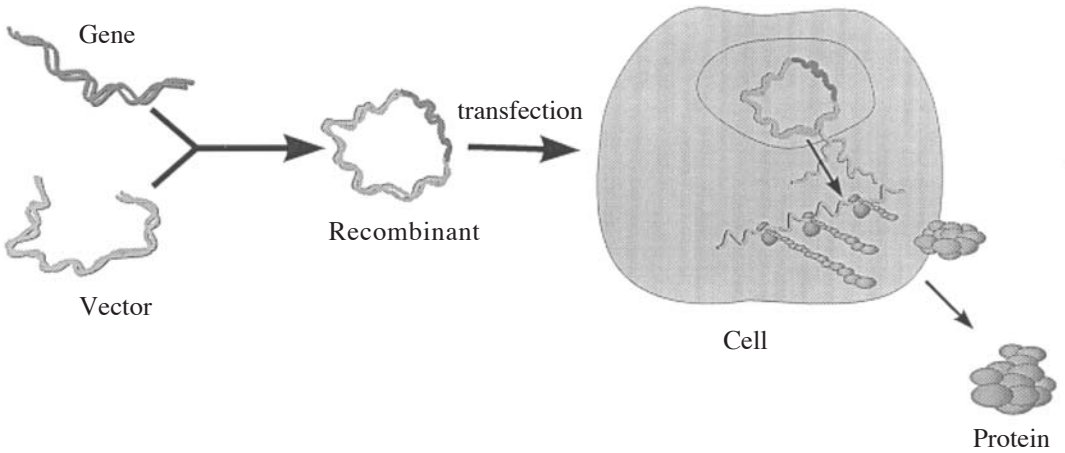

Figure 1: The principle of gene transfer and gene therapy. A particular gene is linked to a vector DNA molecule and introduced into the nucleus of a mammalian or patient cell by one of the available gene transfer techniques. Within the recipient cell, the transferred gene governs the synthesis of the respective gene product, normally a protein which functions inside the cell or is secreted .

gies for gene delivery are employed. First, gene transfer *ex vivo* into patient's cells and immediate retransplantation requiring a reasonable transfer rate (>10%) and high stability of the gene to avoid the necessity of frequent repetition. The second strategy is *in vivo* gene transfer with the preferential targeting of an affected organ. The latter strategy is preferable in most cases. However, none of the described simple transfer techniques can be applied to the *in vivo* situation. Therefore, more advanced tools are required.

Viral vectors

Many types of viruses are very efficient in infecting target cells and delivering genes for expression by the target cells' nucleus [4]. Both small DNA and RNA tumour viruses were studied intensively over the years with regard to their molecular structure and function. They were dissected precisely and viral genes were replaced by foreign genes. Some of these viruses turned out to be well suited for delivery of foreign genes in cell culture and also in some *in vivo* situations (Table III).

Table III: Vectors for gene transfer in vitro *and* in vivo.

Vector	Efficacy		Persistence
	in vitro	*in vivo*	
Viral vectors			
Retrovirus	high	low	long
Adenovirus	high	high	short/moderate
Herpes virus	high	moderate	short/moderate
Adeno-associated virus	high	low/moderate	long
Baculovirus	high	low/moderate	short/moderate
Non-viral vectors			
Liposomes	moderate	low	short
Receptor-targeting	high	low (?)	short

Retroviral vectors

Retroviruses are RNA tumour viruses which are widespread among mammals. They transduce cellular genes with high efficiency. Retroviral oncogenes are derived originally from the host cellular genome where they have been picked up in the course of viral integration and replication. The unique property of retroviruses is their efficient integration into the host genome. This integration is specific on the viral side but rather nonspecific on the host side [5]. The viral genome is linear with flanking long terminal repeat (LTR) sequences which serve as control elements for replication, integration and gene expression. Most retroviruses, including the prototype vector Moloney mouse leukemia virus, have just three genes between the flanking LTRs which code for the envelope proteins and the viral reverse transcriptase. These genes could be removed from the viral genome and replaced by a foreign gene, provided the viral genes are present in the same cell for generation of recombinant viruses. So-called packaging helper cells were generated in which the viral genes were integrated stably [6]. These packaging cells produce empty viral particles. Upon transfection of a recombinant DNA consisting of the gene of interest flanked by the viral LTR sequences plus a short sequence required for recognition by the packaging machinery (packaging signal=psi) recombinant viruses are generated in culture and are produced continuously. The supernatant of such a culture can be used to infect a variety of target cells. The titres of viral supernatants routinely are between 10^5 and 10^6 infectious units per ml [5, 6]. Depending on the origin of the virus envelope gene, the virus can either infect only mouse cells (ecotropic), only non-mouse cells (xenotropic), or almost all mammalian cells (amphotropic). Using the latter type of virus a large number of model gene therapy experiments have been carried out *ex vivo*. Some of them will be described later. The efficiency of integration into the host genome is considered to be the major advantage of this type of vector because it guarantees long-term stability of the gene [5, 6]. The potential disadvantage of inactivating some cellular function or activating an oncogene is regarded as a minor problem because it has not been observed in experimental studies so far. There are two major requirements which make this type of viral vector less suitable for application *in vivo*. First, it can infect only dividing cells [7] which are rarely encountered *in vivo* and second, a mouse retrovirus gets neutralized by the human complement [8]. Strategies for circumventing these problems are currently being developed.

Adenoviral vectors

As opposed to retroviruses, adenoviruses efficiently infect resting cells, are not neutralized by complement and do not integrate their DNA into the host genome [9]. The first two properties make the virus a good candidate for *in vivo* gene delivery. In fact, this vector type, which is derived mainly from the non-malignant human adenovirus type 5, can infect most animal tissues *in vivo* depending on the route of administration [10]. Intravenous (tail vein) injection leads to efficient targeting to the liver in mice and rats. The stability of the transferred gene is related to the intensity of replication because it is only attached loosely to the chromatin and becomes increasingly lost during further replications. Average periods of gene expression are between one and three weeks. Thus, repeated application is required, e.g. for treatment of genetic diseases. However, this most likely would be impossible due to a humoral immune response. Moreover, the use of adenoviral vectors for gene transfer therapies is linked currently to both toxicity problems and cellular immune responses to newly synthesized viral proteins in infected cells [11]. Toxicity can be minimized by using suboptimal titres for infection. At 100 infectious units per cell, where normally 80-100% gene transduction is achieved, some degree of toxicity is observed. Therefore, one could use 20-50 infectious units per cell with a transduction rate of about 50%. Cellular mediated immune responses, however, are a more serious problem. These could be avoided by eliminating most of the viral proteins from the vector. Since the viral genome codes for several proteins which are dispensable in *cis* position and can be provided in *trans* by a helper cell line, efforts are underway to develop more advanced helper cells. First generation standard adenoviral vectors are deprived of only the E1 gene, which is essential for viral replication [12]. This function is provided by the replication helper cell line 293 in which replication-deficient vectors are produced normally [12]. Unfortunately, the viral proteins responsible for eliciting host cellular immune responses have not been identified. Although mutants in the E2 region are associated with the prolonged survival of infected cells, the responsible mechanism remains unclear [13]. More work is required to characterize the viral functions and proteins involved in antigen presentation, toxicity, and stability of adenoviral vectors.

Adeno-associated virus

A small single stranded DNA virus which normally requires the presence of an adenovirus for its own replication has been identified. It is called adeno-associated virus (AAV). This virus was found to integrate with high accuracy into a certain sequence in human chromosome 19 which has no obvious function [14]. This integration specificity makes this virus more attractive than retroviruses to achieve stable gene transfer without side-effects on the genomic level. However, it was shown recently that the integration specificity is lost in recombinant viruses. Thus, the viral genome structure is somehow involved in the specific integration mechanism. The resulting random integration into the genome may be a useful property if the virus has other advantages over retroviruses. One possible advantage is the recently discovered ability for this virus to infect preferentially S-phase cells as opposed to mitotic cells as is the case with retroviruses [15]. The responsible mechanism probably does not require total DNA replication, but only DNA repair [16], which is normally most active in S-phase cells. DNA repair synthesis can be induced by DNA damaging agents administered at low concentrations as done in cancer chemotherapy. One disadvantage shared by AAV and retroviruses is the low titres of infectious viruses obtained which disclose application *in vivo* so far.

Other viral vectors

Besides the three types of viral vectors already described, there are other viruses which might be useful for gene delivery under certain circumstances or for application to particular tissues. Until now, tissue tropism of viruses has not been applied rigorously as a criterion to find new vectors. One potential virus which is increasingly popular for application to the central nervous system is herpes simplex [17].

Our group has demonstrated recently that the insect virus AcNPV (baculovirus) can efficiently and specifically target hepatocytes *via* a receptor-dependent endosomal pathway [18]. This unexpected finding suggests that other viruses might exist which use specific surface molecules as entry sites into cell. Baculovirus has a large genome and can accommodate much bigger pieces of foreign DNA than all other viral vectors already studied. This allows for the delivery of complete genes of 30kb and more. It does not express its own genes in mammalian cells and should, therefore, not induce a cellular immune response by the host. Unfortunately, it is neutralised by complement. Routes for circumventing this problem are currently under investigation. Hepatitis viruses have a strong tropism for hepatocytes but their genomes are fairly small and thus probably not suitable for the accommodation of therapeutic genes. Thus, the ideal viral vector has still to be found or generated by the extensive modification of existing vectors.

One alternative may be the use of viral envelopes or coat proteins. These can be produced by the overexpression of their respective genes, e.g. in insect cells. This has been done recently for hepatitis B surface antigens [19] and for polyoma virus coat proteins [20]. Whereas the former were shown to form almost authentic viral particles and to bind specifically to hepatocytes [19], the latter formed pseudovirions which were efficient in the nuclear delivery of genes [20]. Thus, the use of virus-derived components for the generation of artificial gene transfer vehicles appears promising.

Non-viral vectors

In the light of problems which are encountered with viral vectors, particularly in relation to the immune system, it is a good idea to look for alternative delivery systems, e.g. vectors which are constructed artificially (Table III).

Liposomes

Liposomes have been known for some time as suitable drug carriers. They are classified according to their chemical charge at physiologic pH, i.e. as anionic, neutral and cationic. Whereas the first two types were designed for inclusion of drugs and shown to be useful for the inclusion and protection of DNA, the latter are capable only of complexing *via* their cationic moiety with DNA's phosphate backbone, thus creating an almost neutral net charge. This latter type of liposome is taken up more efficiently by cultured cells. *In vivo,* the first two types are taken up almost exclusively by the immune system's phagocytic cells, predominantly in liver and spleen [21]. Cationic liposomes reach most tissues, particularly epithelia, upon injection into the tail vein of mice. The efficiency of gene delivery by this method remain fairly low. The great advantages, however, over most viral vectors are their low immunogenicity and their ability to package large pieces of DNA [21]. The first feature made them particularly attractive for some current clinical studies (see below).

Receptor targeting

The idea of targeting a vector to a particular cell type *via* unique receptors is very appealing. This concept was introduced originally by Wu *et al.* [22] and was modified and extended later by Birnstiel and coworkers [23]. The basic principle involves wrapping DNA with a polycationic substance like the polypeptide polylysine and complexing it with a ligand for a particular receptor. The original approach targeted the asialo-glycoprotein-receptor and used asialo-orosomucoids as ligands. Hepatocyte-specific uptake was demonstrated *in vitro* and *in vivo*. However, the transfer rate was very low. By exploring the transferrin receptor as a target, Birnstiel and coworkers demonstrated that endosomal entrapping made receptor tageting inefficient; when they applied inactivated adenovirus at the same time they obtained dramatic increases in gene transfer efficiencies due to endosome disruption by adenovirus [24]. Since the use of adenoviruses with these particles or their coupling to them reduces their versatility, alternative ways for endosome disruption must be found. One alternative is the use of influenza haemagglutin [25]. Major problems related to the particles' large size (>100 nm) was solved recently; by carefully adjusting the salt

concentration particle size was reduced significantly (10 nm) [26]. Thus there are new opportunities to apply receptor targeting to particular tissues *in vivo* in the near future. The use of polypeptide components in receptor-targeting particles, however, still poses a problem for repeated application due to the particles' inherent immunogenicity.

Alternative strategies

Transfer of naked DNA

Although it is difficult to do on a large scale, the microinjection of naked DNA into the nucleus has proven to be very efficient for gene transfer *in vitro*. Thus, alternative ways of delivering DNA to the nucleus of living cells have been possible. Two different procedures use high pressure for delivery. The gene gun technology is based on small gold or tungsten particles on which the DNA has been condensed; the particles are delivered under high pressure to a larger number of cells within a certain area; since the whole cell is bombarded with a dense stream of particles, the percentage of nuclei which are reached is quite high [27]. The jet injection technology uses DNA in solution under higher pressure [28]. These two techniques are suitable for application *in vivo*.

A surprising finding was the demonstration of successful gene transfer to tissues *in vivo* by direct syringe injection [28]. This procedure is particularly applicable to muscle tissues which have a low content of nucleases. Here, a certain percentage of injected DNA reaches the nucleus and is expressed. Antibodies against several antigens have been raised in mice by this technique. This phenomenon is now used intensively for direct immunization against certain antigens and could potentially be useful for cancer immunotherapy.

The EBV replicon

Besides retroviruses and AAV, all non-viral and viral vectors suffer from the problem of short-term stability of transferred DNA within the target cell. Indeed, for a large number of potential applications, it would be preferable to achieve sustained expression for a longer period of time or even throughout life. Stable integration into the genome occurs rarely. The extrachromosomal establishment of a vector is an attractive alternative. The replication unit of the Epstein-Barr virus (EBV) has been employed successfully to this end. The replication unit consists of oriP (the origin of latent replication) and a gene for the replication-supporting protein EBNA1 (Epstein Barr Nuclear Antigen 1) [29]. Plasmids containing these two components of the EBV establish themselves as self-replicating episomes in a variety of cell types when they are delivered by one of the methods previously described. EBV replicons could be part of liposome-based or a receptor-targeting vector. This approach permits the establishment of the gene for several months or even years. One major concern is the presence of EBNA1, the function of which is still not understood completely. Obviously, this gene product is responsible for the escape from immune surveillance [30]. This could be an advantage with regard to the stability of the vector-containing cells but could be disadvantageous in cases of malignant changes. At least, it is known that a Gly-Ala repeat sequence within the protein is responsible for the immune effect [30] and can be removed without influencing the replication-supporting function. Thus, there are good prospects for the use of the EBV replicon for gene therapy vectors. It could even be combined with viral vectors like adenovirus or baculovirus to generate an episomal system based on these efficient delivery vectors.

Antisense technology

Genetic technology has shown that several functions, particularly the translation of messenger RNAs (mRNAs), can be inhibited by antisense molecules [31]. A single stranded DNA or RNA which is complementary to the sequence of the mRNA forms hybrid molecules by base-pairing. Antisense molecules can be transcripts of the gene of interest (or parts of it) cloned in the opposite orientation behind a suitable promoter. This kind of antisense gene may be useful for continuous inhibition of the expression of the target gene. The extent of inhibition normally does not exceed

50-70% even if the antisense gene is expressed at levels 10 times higher than that of the target gene. Stronger inhibition can be obtained by using high amounts of synthetic antisense oligonucleotides 15-18 nucleotides in length. Modifications of the original oligonucleotide backbone structure render them resistant to nuclease degradation and allow for prolonged activity *in vitro* and *in vivo*. Antisense oligonucleotides are used widely for experimental purposes to inhibit the expression of genes but their high degree of toxicity prevents their broader application for therapeutic purposes. One clinical trial for cancer treatment using antisense oligonucleotides for the inhibition of oncogene expression is underway currently [32]. The level of repression obtained with antisense oligonucleotides can approach 90% which can still be too little, e.g. for inhibiting expression of HIV or HCV genes.

Another finding of the last few years is useful in this respect. Some organisms have evolved self-cleaving RNA molecules. The catalytic domain of these so-called ribozymes resides in structures called either hammerheads or hairpins, depending on the actual structure. Ribozymes have a very short recognition sequence of only three nucleotides which appear several times within a particular mRNA. It was shown that these short hammerhead or hairpin structures can be removed from the original RNA and placed in the context of an antisense RNA in a position where a potential cleavage site is present in the target RNA, thus allowing the recombinant ribozyme to cleave the target RNA to which the antisense part is complementary. Ribozymes can be produced synthetically or expressed as part of an antisense gene. The latter is probably the most powerful technique for the inhibition of gene function. Using an optimized expression cassette and a target-selected ribozyme from a library of 10^{10} different ribozymes, we have achieved 99.9% inhibition of a highly expressed growth hormone gene [33]. New strategies for the retroviral delivery of ribozymes to the site of function within the cell will allow for their efficient *in vivo* function [34].

A new application of the ribozyme technique is currently emerging. This strategy aims to repair mutant gene products by cutting the respective mRNA in a certain position upstream of the mutation and fusing the intact 5'-end of the mRNA to an intact 3'-end which is part of the ribozyme structure [35]. This so-called *trans*-splicing technique is particularly applicable where inactivation of the mutant gene product is required.

Preclinical models

The first steps in the application of gene therapy are a series of successful experiments in cell culture to demonstrate the approached theoretical efficacy. An essential step preceding clinical application of the technology is the preclinical assessment of various parameters. A step which is absolutely required is toxicity testing. This is done normally in mice or rats. However, it would be preferable to have an animal model for the actual disease in order to demonstrate the proposed approach. Unfortunately, only a small number of natural disease models are available such as the hypercholesterolaemic New Zealand White Rabbit (Watanabe rabbit). However, the new age of mouse genetics based on specific gene inactivation by homologous recombination has led to the generation of a large number of disease models within the last few years [36]. Several basic strategies for gene therapy have thus been tested extensively in animal models before they were applied in clinical trials. Only a few recent examples will be discussed briefly.

The haematopoietic system has received most of the attention of gene therapists. The possibility of treating either lymphocytes or stem cells *ex vivo* with a certain vector and reinjecting into the patient is very appealing. The strategy is rather simple (Fig. 2). The only new step for the clinician experienced in bone marrow transplantation is the incubation of purified cells with the vector, in this case preferably a retroviral vector. Retroviral gene transfer of a variety of genes has been investigated by a large number of groups in small mammals and primates [37]. The overall outcome of these studies was fairly high (20-40%) transfer rates in peripheral lymphocytes but that of stem cells is rather low (2-10%). After enrichment of positive cells, a sustained expression of the respective gene (mainly ADA) was detected and generation of replicating viruses could not be observed.

These results paved the way for the first clinical studies of gene therapy of ADA deficiency in the haematopoietic system (see Fischer *et al.*; in this issue, pages 25-34).

Model experiments for the treatment of cystic fibrosis were initiated originally with the cottontail rat, an established model for testing adenoviral infection. Adenoviral gene delivery to the lung was optimized in these animals originally using the alpha-1-antitrypsin gene [38] and later with the CFTR gene. Functional studies were possible only with the newly created CFTR gene knockout mice in which the liposome-mediated gene delivery was tested [39]. Transient gene expression at low-to moderate rates was already suggesting that the clinical application would not lead to a cure at this stage (see Coutelle; in this issue, pages 15-24).

Gene transfer targeting liver has been tested quite intensively with almost every transfer system available [40]. Most are useful in hepatocyte cell cultures. Many efforts have been directed to use the retroviral vectors. Wilson and coworkers developed protocols for the *ex vivo* infection of hepatocytes taken from a collagenase-treated liver lobe and their subsequent transplantation by infusion into the portal vasculature; with maximal transduction efficiencies of 40%, some 3% of the liver of a rabbit consisted of transduced cells after a few days leading to a significant reduction of serum cholesterol [41]. This procedure was employed recently in a clinical protocol (see below). There are two ways for optimizing this protocol. The retroviral transduction rate recently has been increased considerably by treating isolated hepatocytes with hepatocyte growth factor [42] and methods for the stimulation of liver regeneration have been developed [43], which should improve the efficacy of hepatocyte transplantation.

Since this *ex vivo* procedure always will be very complicated, *in vivo* gene transfer has been tried in various animals involving partial hepatectomy as a means to stimulate cell division, an essential prerequisite for retroviral infection. The rate of gene transduction did not exceed 5% with this

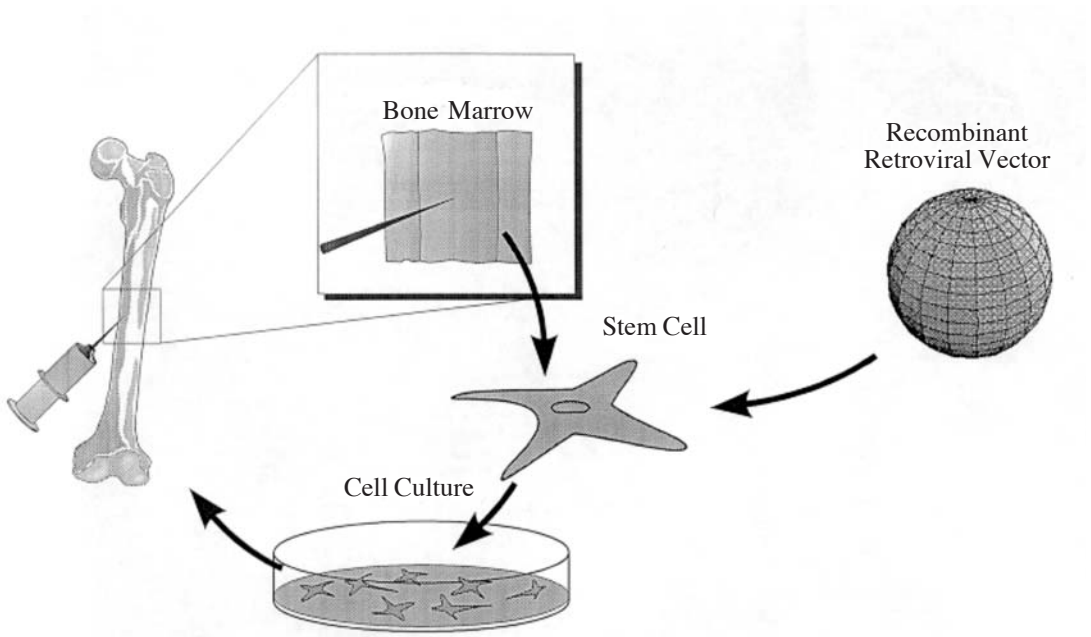

Figure 2: Strategy for ex vivo *gene therapy in bone marrow stem cells. Stem cells are purified from patient's marrow, cultured for a short period of time and infected with a retroviral vector. After some hours, the stem cells are grafted to the patient .*

procedure in combination with portal vein injection either, but vascular exclusion seems to increase the efficiency of gene transfer by 4-10 fold in the rat [44], most likely due to the prevention of viral neutralisation by complement. An important success was achieved recently with the portal route of infection. A haemophilic dog sustained expression of subtherapeutic levels of factor IX for over 9 months; this led to a drastic reduction of blood clotting time [45]. All attempts have shown clearly that gene therapy in the liver is feasible if the problem of efficient gene delivery can be solved. Using adenoviral vectors, almost 100% gene transduction to the liver was obtained in mice after intravenous injection and therapeutic levels of alpha-1-antitrypsin were produced for a short period of time in an animal model of alpha-1-antitrypsin deficiency [46].

Another exciting gene therapy approach designed to provide a continuous supply of normally secreted proteins was taken by Danos and coworkers [47]. These authors developed so-called organoids from primary fibroblasts transduced *ex vivo* with a particular gene using a retroviral vector. The transduced cells were grown on collagen and synthetic polymer fibres coated with heparin and basic fibroblast growth factor to stimulate angiogenesis. Small organoids developed within a few days and were implanted in the peritoneal cavity. Vascularized neo-organs rapidly developed and provided a suitable environment for long-term cell survival. This system was applied first to the delivery of β-glucuronidase which is missing in mucopolysaccharidosis type VII (Sly syndrome). After having successfully treated a mouse mutant model for Sly syndrome, these investigators demonstrated recently in dogs that organoids can function actively for at least 1 year without local inflammation or other side effects. The level of ß-glucuronidase produced amounted to only 1-3% of normal levels observed in dogs [47]. This technology should be applicable to the treatment of a number of lysosomal storage diseases, as well as haemophilia and alpha-1-antitrypsin deficiency (see Poenaru; in this issue, pages 35-44).

Finally, important progress has been made recently towards the prevention of restenosis after balloon catheter treatment. The major problem in this condition is hyperplasia of smooth muscle cells (SMC) at the injury site. This phenomenon can be prevented efficiently by the downregulation of cell cycle activity. The local delivery of an inhibiting substance is required in the particular region of an injured blood vessel. This can be achieved *via* the balloon catheter during angioplasty. Antisense oligonucleotides directed against the mRNAs of positive regulators of cell division like the cyclins have proven to be efficient in animals [48]. Most recently, the retinoblastoma tumour suppressor gene Rb was transduced by an adenoviral vector both into the rat carotid and into the porcine femoral artery; this resulted in the efficient prevention of SMC proliferation and neointima formation [49]. This kind of application of gene therapy is probably most promising at the current stage of development because only a single treatment is required to prevent hyperplasia and the effect is needed only for a few days. Once proliferation of normal SMC is blocked, endothelial cells can regenerate and close the injured vessel. Clinical application of this procedure is in preparation (Leiden, personal communication).

Clinical trials

The first official clinical trial for gene therapy was initiated in September 1990 by Blaese and coworkers for the treatment of ADA deficiency [3]. This disease, albeit rare, was particularly suited as a target for the first trial not only because peripheral lymphocytes could be used but also because the actual level of gene expression is not critical. Expression at 10% of normal levels is sufficient for a therapeutic effect. The first trial involved two girls treated in seven sessions over half a year with purified homologous T-lymphocytes infected with a modified retrovirus. In the course of this treatment, the level of ADA-positive T-lymphocytes increased from 10% to 40% and remained at these levels for almost half a year suggesting a positive effect of the gene on lymphocytes survival. The patients were challenged with a number of mild infections showing that their immune response was functioning to some extent. However, they also were treated with polyethyleneglycol (PEG)-coupled purified ADA en-

zyme, as before gene therapy; it is thus difficult to judge the treatments' success. In order to provide a permanent cure, gene transfer was carried out subsequently in bone marrow stem cells. Bordignon in Milano [50] and Valerio in Holland [51] also have carried out clinical trials for the treatment of ADA deficiency. Bordignon has treated stem cells and T-lymphocytes in parallel with two slightly differing vectors in order to investigate the relative contribution of the two cell types to ADA positive peripheral lymphocytes. The results of the Blaese and Bordignon studies were recently published [52, 53].

Another clinical trial is that by Wilson and coworkers (Ann Arbor, MI) for familial hypercholesterolaemia initiated in June 1992 [54, 55]. A 28 year female with a missense mutation in her LDL receptor gene at amino acid 66 and with a total serum cholesterol concentration of 545 mg/dl (LDL of 482 mg/dl, HDL of 43 mg/dl) was treated. First, the left lateral segment of the liver (15% of total liver mass) was removed. A catheter was inserted into the inferior mesenteric vein and the distal end of the catheter was brought through the incision to allow for later infusions. The resected liver (250 g) was perfused with collagenase leading to the release of 3.2×10^9 hepatocytes. These were seeded in 800 10 cm plates. Two days later, the cells were incubated with the retroviral vector carrying the LDL receptor gene for 12-18 hours. Following this treatment, 20% of the cells took up LDL. Some 2×10^9 cells were harvested in three aliquots and each aliquot was infused manually at 4 hour intervals directly into the catheter over 30 min periods. A significant percentage of positive cells were detected by hybridization 4 months later. The LDL level was reduced to 404 mg/dl, and even further to 356 mg/dl after lovastatin treatment four months later. This was a significant effect but of very limited therapeutic value.

Clinical trials phase I were also carried out for the treatment of cystic fibrosis [56] (see Coutelle; in this issue, pages 15-24).

Two strategies for the enhancement of cancer immunotherapy were developed by Rosenberg *et al*. and applied in clinical trials for the treatment of melanoma [57, 58]. Both were based on transfer of the gene coding for interleukin 2 (IL-2). In the first one, the IL-2 gene was transferred into tumour-infiltrating lymphocytes using a retroviral vector. In the second, the IL-2 gene was transferred into the patient's tumour cells which were used as an immunogen afterwards. Since the latter strategy is more flexible, it has found wide application recently for the treatment of a variety of tumours. As phase I trials are generally carried out with patients in the terminal stage, it is not surprising that cures have not been described. However, considerable regression of tumours has been reported at several symposia.

Another strategy for gene therapy was developed by Blaese and coworkers which was applied first to the treatment of brain tumours [59] but can be applied to all types of solid tumours with some modifications. The principle is as follows: the thymidine kinase (tk) gene of herpes simplex virus converts the non-toxic substrate analogue ganciclovir to a triphosphate product which is toxic to growing cells; these incorporate this product into DNA. If a retrovirus is used as the vector, it infects only growing cells. After gene transfer, ganciclovir is added and kills growing cells selectively. Interestingly, non-transduced tumour cells in the vicinity of the tk-positive cells also are killed. This so-called bystander effect is due probably to the transport of ganciclovir-triphosphate *via* gap junctions. Normal resting cells are not affected. Thus, this gene therapy protocol, dubbed "suicide strategy", kills tumour cells by highly selective chemotherapy. Since retroviruses are inactivated by complement, this protocol was applied first to the treatment of brain tumours (glioblastomas). As high titres of retroviral vectors are difficult to obtain, vector-produ-

Table IV: Clinical studies on gene therapy in the USA (taken with permission from Culver [60]).

Year	Cancer	Genetical	AIDS	Auto-immune	Circulation	Total
1990	1	1	0	0	0	2
1991	2	1	0	0	0	3
1992	8	3	1	0	0	12
1993	18	7	4	0	0	29
1994	18	7	1	1	1	28
1995	8	2	3	0	0	13
Total	55	21	9	1	1	87

cing cells were injected into the tumour from where virus was released continuously over a period of several days. After successful experiments in animal models this strategy was applied to 10 patients in its first clinical trial. This protocol has been adapted to that of other tumors and likely will be modified, e.g. for the use of adenoviral vectors.

Within the last 5 years about 100 clinical protocols have been approved by the Recombinant DNA Advisory Committee (RAC) in the USA. A summary of the trials for different disease groups is given in table IV. Even if most of these trials do not lead to a therapeutic success, they will contribute to the establishment of gene therapy as a major component of molecular medicine and to the development and implementation of more advanced protocols. Table V gives an overview of the current strategies.

Table V: Present strategies for gene therapy.

Diseases	Vector	Delivery	Gene
ADA deficiency	retroviral	*ex vivo*	ADA
LDL receptor	retroviral	*ex vivo*	LDL-R
Mucoviscidosis	adenoviral	*in vivo*	CFTR
	liposomes	*in vivo*	
MPS	not available		various
Haemophilia	not available		F. VIII/IX
Melanoma	retroviral	*ex vivo*	IL-2,3, etc.
Brain tumours	retroviral	*in vivo*	tk
Lung carcinoma	adenoviral	*in vivo*	p53
Restenosis	oligonucleotide	*in vivo*	antisense G1-cyclins
	adenoviral	*in vivo*	Rb, p16, p53
AIDS	ribozyme	*in vivo*	HIV RNAs

Perspectives

This introduction to the field of gene therapy makes the point that despite the justified excitement about the progress with techniques and vectors in *in vitro* and animal systems, there is still a long way to go before any one disease will be treated successfully and routinely. However, if one considers that the first genes were isolated only 20 years ago and that efficient gene transfer techniques have been available for only little more than 5 years, there is hope that the first generation vectors and strategies will be replaced soon by more advanced ones with higher chances for success in clinical applications.

Key principles of gene therapy have proven feasible and safe in almost 100 studies. A major breakthrough is predictable if gene transfer to fetuses is established. This would not only prevent the onset of a disease but would contribute to the immunotolerance of the newly acquired antigens. Moreover, a relatively small number of treated cells would expand and contribute significantly to the function of an organ like the liver.

Gene therapy in germ cells is considered by most people not to be required or to be unethical. Considering the current state of the art of the technology, this is definitely correct. However, some medical indications might require the exploration of this hitherto tabu field for medical intervention [61]. If this ever comes, very convincing evidence for the overall safety of gene targeting has to be shown. Nevertheless, we have now entered an exponential growth phase of the field of gene therapy that is characterized by great technical advancements [62].

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