Adenoviral Vector-Mediated Gene Transfer for Human Gene Therapy

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Abstract: Human gene therapy promises to change the practice of medicine by treating the causes of disease rather than the symptoms. Since the first clinical trial made its debut ten years ago, there are over 400 approved protocols in the United States alone, most of which have failed to show convincing data of clinical efficacy. This setback is largely due to the lack of efficient and adequate gene transfer vehicles. With the recent progress in elucidating the molecular mechanisms of human diseases and the imminent arrival of the post genomic era, there are increasing numbers of therapeutic genes or targets that are available for gene therapy. Therefore, the urgency and need for efficacious gene therapies are greater than ever. Clearly, the current fundamental obstacle is to develop delivery vectors that exhibit high efficacy and specificity of gene transfer. Recombinant adenoviruses have provided a versatile system for gene expression studies and therapeutic applications. Of late, there has been a remarkable increase in adenoviral vector-based clinical trials. Recent endeavors in the development of recombinant adenoviral vectors have focused on modification of virus tropism, accommodation of larger genes, increase in stability and control of transgene expression, and down-modulation of host immune responses. These modifications and continued improvements in adenoviral vectors will provide a great opportunity for human gene therapy to live up to its enormous potential in the second decade.

INTRODUCTION

Gene therapy, by definition, utilizes directed gene transfer to treat disease. Many believe that it may hold the potential to revolutionize medicine. Gene therapy's great promise comes largely because therapies aim to treat what causes the disease, whereas most current approaches only relieve symptoms. The first clinical gene therapy study emerged ten years ago (Blaese et al., 1995). Early results on the clinical efficacy of gene therapies were somewhat disappointing, largely because the available gene transfer vectors were inadequate (Wadman, 1995). As vector technology has improved significantly, the clinical benefits of gene therapy have been clearly demonstrated (Mountain, 2000). With the completion of human genome project and identification of more disease-causing genes, there is now every prospect that the second decade may see gene therapy live up to its enormous potential. In this review, we will briefly summarize the current status of gene therapy trials in the United States, and then we will focus on the progress, problems, and prospects of adenoviral vector-mediated gene transfer in animal and clinical studies.

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CURRENT STATE OF HUMAN GENE THERAPY

The first gene transfer in clinical study was initiated in 1990 (Blaese et al., 1995) and since then over 400 gene therapy protocols have been submitted to or approved by the National Institutes of Health (NIH) in the United States (http://www4.od.nih. gov/oba and Human Gene Marker/Therapy Clinical Protocols, 2000). As summarized in (Table **1**), almost all clinical studies involve gene addition rather than the correction or replacement of defective genes, which is technically more challenging. Thus far, all clinical protocols involve gene transfer to exclusively

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Table 1. Summary of Gene Therapy Protocols Approved by or Submitted to the NIH

Clasified by Disease	# of Protocols	Classified by Vector	# of Protocols
Cancer	280	Retrovirus	157
AIDS/HIV infection	35	Adenovirus	110
Genetic diseases	47	Adeno-associated virus	11
Cardiovascular diseases	33	Vaccinea virus	29
Rheumatoid Arthritis	2	Herpes simplex virus	3
Multiple Sclerosis	1	Plasmid DNA	35
Acute hepatic failure	1	Liposome/lipids	53
Others	11	RNA	5
Total	410	Peptide-mediated	3
Classified by Delivery Route	**# of Protocols**	Condensed DNA particles	2
In Vivo	238	Electroporation	2
Ex Vivo	172	Total	410

somatic cells rather than germ line cells; the latter has been the subject of considerable ethical debate. Somatic gene therapy can take place *ex vivo* or *in vivo*. In the *ex vivo* approach, target cells are removed from patients for gene transfer and, thus, the therapeutic entity comprises the engineered cells. Although the *ex vivo* gene transfer offers more efficient gene transduction and easier propagation for generating higher cell doses, it has the obvious disadvantages of being patient-specific as a result of immuno-genicity and more costly because cell culture manipulation adds manufacturing and quality control difficulties. On the contrary, the *in vivo* approach involves direct administration of gene transfer vector to patients. It is therefore not patient specific and potentially less costly.

As indicated in (Table **1**), clinical studies are being carried out in a wide range of diseases (Human Gene Marker/Therapy Clinical Protocols, 2000). Of approximately 400 clinical protocols involving gene transfer approved by the regulatory authority, approximately two-thirds of trials are directed at cancer therapy, most of the rest at inherited monogenic diseases (e.g., cystic fibrosis and hemophilia) and infectious diseases (mostly AIDS/HIV infection). This distribution may largely reflect the lack of effective alternative therapies and hence the likelihood of regulatory approval. Most of early clinical trials involved cells engineered *ex vivo* with retrovirus expressing reporter genes and aimed to assess pharmaco-kinetics and biodistribution of the engineered cells in patients with cancer or inherited monogenic disorders. However, almost all of recently approved protocols have therapeutic intent and most protocols focus on destruction of diseased cells rather than long-term restoration of the defective or missing genes. This trend has understandably resulted from limited choices of gene delivery vectors and a lack of long-lived expression systems.

There are three major approaches for gene delivery: viral, non-viral and physical. The most common physical methods involve needle-free injectors and electroporation. Non-viral approa-

ches usually fall into three categories involving 1) naked DNA molecules; 2) DNA complexed with cationic lipids; and 3) particles comprising DNA condensed with cationic polymers or liposomes (Wolff and Trubetskoy, 1998). Many different viruses are being adapted as gene transfer vectors, but the most advanced ones are adenovirus, retrovirus (including recently developed Lentivirus) and adeno-associated virus (Robbins *et al*., 1998). Substantial effort has also been devoted to the development of poxviruses or vaccinia viruses and herpes simplex virus (Sanda *et al*., 1999; Krisky *et al*., 1998) As summarized in (Table **2**), the leading viral vectors generally give the most efficient gene trandsduction, while their main disadvantages concern their limited capacity for accommodating large transgenes, immunogenicity, and manufacture of high quality vector stocks. Non-viral approaches give less efficient transfection and more transient expression, but have no insert-size limitation, are less immunogenic and easier to manufacture. Physical methods give inefficient gene transfer and have a limited range of applications.

More than 3,500 patients have been enrolled for gene therapy clinical trials. In general, good safety results have been observed in most Phase I/II trials, but the outcome of gene transfer efficacy and the therapeutic benefits have been disappointing. However, there are several notable exceptions. As indicated in (Table **3**), several protocols have shown signs of promise and are currently conducting phase II trials (Human Gene Marker/Therapy Clinical Protocols, 2000; Baumgartner *et al*., 1998; Swisher *et al*., 1999; Wagner *et al*., 1998; Stopeck *et al*., 1997; Boussif *et al*., 1996; Rogulski *et al*., 2000; Ram *et al*., 1997). Nevertheless, the lack of efficacy data for most clinical trials prompted the NIH to appoint an expert committee in 1995 (Wadman, 1995). The committee eventually recommended that resources should be diverted away from premature clinical trials with inadequate vectors and applied to basic studies on gene transfer and gene expression technologies that are more relevant to preclinical testing. This decision has led to more productive investigations on vector improvement and gene transfer efficacy. The major features of commonly used vector systems are compared in (Table **2**).

Table 2. Comparison of Common Gene Transfer Approaches

Features	Adenoviral	AAV	Retroviral	Lentiviral	HSV	Non-viral
Host range	broad	broad	restricted	broad*	restricted	broad
Transducing efficacy	very high	high/moderate	low	low	moderate	very low
Chromosomal integration	no	yes/no	yes	yes	no	no
Duration of expression	weeks-months	long-term	long-term	long-term	days	days
Construction procedure	easy	established	established	difficult	difficult	varied
Transgene size	5 - 36 kb	4 - 5 kb	4 - 5 kb	8 - 9 kb	large	unlimited
Vector yield (titer)	high ($>10^{11}$)	low ($<10^{9}$)	low ($<10^{7}$)	low ($<10^{6}$)	high (10^{10})	high
Host cell proliferation	not required	not required	required	not required	not required	not required
Regulatable expression	available	available	possible	possible	difficult	available
Immune responses	high/low**	low/rare	rare	rare	high	low

Notes *VSV-G pseudotyped HIV vectors
*reduced responses against gutless vectors

Table 3. Current Protocols in Phase II Clinical Trials

Disease	Delivery vehicle	Targeted cells	Transgene	Rationale	Reference
Cancer	adenoviral	cancer cells *in vivo*	p53	inducing apoptosis	10
Cancer	adenoviral	cancer cells *in vivo*	Ad genes	producing lytic viruses	14
Cancer	retroviral	fibroblasts *ex vivo*	IL-12	enhancing tumor immunogenicity	4
Cancer	retroviral	cancer cells *in vivo*	TK	producing toxin-making enzyme	15
Cancer	liposome	cancer cells *in vivo*	HLA B7	enhancing tumor immunogenicity	12
Cancer	liposome	cancer cells *in vivo*	IL-2	enhancing tumor immunogenicity	4
Cancer	liposome	cancer cells *in vivo*	Ad E1A	inducing apoptosis	13
Cystic fibrosis	AAV	airway cell *in vivo*	CFTR	replacing defective proteins	11
AIDS	retroviral	T cells *ex vivo*	chimeric TCR	retargeting killer T cells	4
Limb ischaemia	plasmid DNA	muscle cells *ex vivo*	VEGF	promoting neoangiogenesis	9

Despite some obvious shortcomings, adenoviral vectors remain as one of the most viable gene delivery approaches for human gene therapy.

ADENOVIRUS BIOLOGY

Decades of study on adenovirus biology have yielded a detailed picture of the viral life cycle and the functions of the majority of viral proteins. Adenoviruses are non-enveloped DNA viruses whose capsid is mainly composed of pentons (penton base and fiber monomers) and hexons (Shenk, 1996). The genomes of the most commonly used human adenoviruses (type 2 and type 5) consist of a linear 36 kb double stranded DNA molecule. Both strands are transcribed and nearly all transcripts are heavily spliced. Viral transcription units are conveniently defined as early (encoding E1, E2, E3, and E4), delayed early (encoding transcripts IX and IVa), and late genes, depending upon their temporal expression relative to the onset of viral DNA replication (Shenk, 1996). The early gene products are mostly involved in adenoviral gene transcription, viral DNA replication, host immune suppression, and inhibition of host cell apoptosis (Flint and Shenk, 1997). The late gene transcripts encode proteins necessary for virus assembly. Among the early genes, E1A is the first gene expressed after viral infection and is the most important transcriptional activator for subsequent adenoviral gene expression. The E1A gene products are also involved in viral replication by inducing G1-S transition in the host cells (Flint and Shenk, 1997).

Adenoviral infection is initiated by the high affinity binding of the fiber protein knob domain to cell surface receptors, such as CAR (coxsackievirus and adenovirus receptor) or MHC-I α2-domain, followed by the interaction of the pentons with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin proteins (Bergelson *et al*., 1997; Hong *et al*., 1997). After receptor-mediated endocytosis, the virus escapes from the endosomal compartment to the cytosol and reaches the nucleus where viral transcription and replication begin. The process of viral internalization and release to the cytosol takes about 15 minutes, while transgene expression is detected within 18 hours after infection, reaching a maximal level at 48 to 72 hours post infection (Greber *et al*., 1993). The susceptibility of different cell types to adenoviral infection is largely dictated by the availability of cell surface receptors (Walters *et al*., 1999; Huang *et al*., 1995)

Completion of the virus cycles triggers cell death and the release of virion progeny.

RECOMBINANT ADENOVIRUS VECTORS

The recombinant adenovirus vectors were first used in gene transfer in 1985 (Yamada *et al*., 1985; Ballay *et al*., 1985; Karlsson *et al*., 1986). Recombinant adenoviruses are not capable of replicating themselves as efficiently as wild-type viruses, but are able to replicate efficiently in permissive host cells or packaging lines, such as 293 cells (human embryonic kidney cells transformed by sheared adenoviral genomic DNA) and 911 cells (E1-transformed human embryonic retinal cells) or other packaging lines, in which the E1 proteins are provided *in trans* (Schiedner *et al*., 2000; Fallaux *et al*., 1996; Graham *et al*., 1977). Subsequently, a large quantity of recombinant adenovirus can be produced in 293 cells or other packaging cells and purified by CsCl gradient ultracentrifugation. A virus stock with a titer of 10^{11} to 10^{13} pfu/ml can be routinely prepared.

The high density and complexity of the viral transcription units poses significant difficulties for recombinant manipulation, which therefore is usually restricted to specific regions, particularly E1, E2A, E3, and E4. Thus, the first generation viral vectors are usually recombinant adenoviruses that contain transgenes in replacing E1A and E1B genes. However, these vectors have several apparent limitations in their application as gene delivery vectors. First, these vectors have relatively limited packaging capacity and can only accommodate transgenes smaller than 5 kb (Bett *et al*., 1993). Secondly, the E1-deficient adenoviruses still exert significant cytotoxicity to infected cells. In addition, they incite a marked immune response in infected animals, which in turn, limits the duration and level of transgene expression and complicates the functional analysis.

In the past few years, intensive and substantial efforts have been undertaken to improve the deficiency of the first generation adenoviral vectors, with particular emphasis on accommodating larger transgenes, reducing the cytotoxic effects in host cells, and diminishing the ability to elicit host immune response. For instance, the recently developed adenoviral vectors with double deletions (ΔE1 and ΔE3) or triple deletions (ΔE1, ΔE3 and ΔE4) can accommodate up to 10-kb of foreign DNA (Bett *et al*., 1995; Gao *et al*., 1996; He *et al*., 1998). Another second generation vector with double deletions of E1 and E2 was shown to prolong transgene expression and reduce cytotoxic effects *in vivo* (Engelhardt *et al*., 1994; Engelhardt *et al*., 1994; Schowalter *et al*., 1999; Yang *et al*., 1994). More interes-tingly, by employing a helper virus that provided other essential functional and structural genes *in trans*, the generation of so-called "gutless" adenoviral vector has been recently accomplished (Parks *et al*., 1996; Kochanek *et al*., 1996; Fisher *et al*., 1996). In the gutless vector most of the viral genome is removed to provide room for exogenous DNA. This system has a capacity to accommodate up to 35 kb of foreign DNA and is considered the most advanced vector system to date. Thus, if the helper virus can be completely removed from the recombinant adenovirus stock, the gutless vector may represent one of the most promising gene delivery vehicles. Thus far, it has been reported that the gutless adenovirus has exhibited significantly reduced host immune response *in vivo* and achieved long-term expression of multiple transgenes in a single vector (Schiedner *et al*., 1998; Morsy *et al*., 1998; Morral *et al*., 1998). (Table **4**) summarizes the biological and structural features of currently used adenoviral vectors.

TECHNOLOGICAL ADVANCES IN GENERATING RECOMBINANT ADENOVIRUSES

Traditionally two general approaches have been employed to generate recombinant adenoviruses. The first involves direct ligation of DNA fragments of the adenoviral genome (with deletion of E1-region) to restriction endonuclease fragments containing transgenes (Ballay *et al*., 1985; Rosenfeld *et al*., 1991). Despite the advances made by Mizuguchi and Kay (Mizuguchi and Kay, 1999)??? the difficulty of purifying large viral genomic DNA fragments, the low efficiency of large fragment ligations, and the scarcity of unique restriction sites have made this approach technically challenging. The second and more

Table 4. Currently Used Adenoviral Vectors

Ad Vector	Gene(s) Deleted	Packaging Line	Cloning Capacity	Biosafety Features
ΔE1	E1	E1-expressing cells	3 - 5 kb	residual viral gene expression
ΔE1	E1 and E3	E1-expressing cells	7.5 kb	residual viral gene expression
ΔE1ΔE4	E1, E3 and E4	E1/E4-expressing cells	10 kb	reduced residual viral gene expression
ΔE1ΔE2	E1, E2a/E2b & E3	E1/E2-expressing cells	up to 9 kb	reduced residual viral gene expression
Gutless	all viral genes	helper virus	up to 37 kb	helper virus contamination

widely used method involves homologous recombination in mammalian cells capable of complementing defective adenoviruses (i.e., packaging cells). This prototypic method requires a two vector system, namely "shuttle" and "helper" plasmids (McGrory *et al*., 1988). The shuttle vector usually contains the 5'-end of the adenoviral genome, in which E1A and E1B genes are replaced by a transgene whose expression is driven by its own regulatory elements. The helper vector provides most of the adenoviral genomic backbone but itself is not capable of producing viruses, because it contains either the whole but unpackagable viral genome or most viral genomic backbone with deletion of the E1 and other essential genes. The introduction of both vectors into the packaging cells, through homologous recombination, leads to the generation of recombinant adenoviruses, in which the transgene is incorporated into the viral genome by replacing the E1A and E1B genes. Although successful, this method has been proven laborious and time-consuming, mostly because the efficiency of homologous recombination is extremely low in mammalian cells.

To improve recombination efficiency between transgene-containing shuttle vectors and adenoviral backbone vectors, Ketner, *et al* were the first to exploit the highly efficient homologous recombination machinery in *Saccharomyces cerevisiae* to produce infectious yeast artificial chromosome (YAC) clones containing human adenoviral genome (Ketner *et al*., 1994). Since then, there have been substantial efforts in developing more efficient and simplified systems to generate recombinant adenoviruses (He *et al*., 1998; Chartier *et al*., 199). Currently, one of the most widely used techniques generates recombinant adenovirus vectors in certain strains of *E.coli* cells, which are proficient in generating stable recombinants under proper selective growth conditions (He *et al*., 1998). Alternatively, efficient recom-bination mediated by Cre recombinase/loxP site-specific recombination system has also been reported(Hardy *et al*., 1997; Ng *et al*., 1999). By improving recombination efficiency, the technological advances of the past few years have circumvented the rate-limiting step in recombinant adenoviruses production. These steps make it possible to generate a large quantity of adenoviral vectors in an efficient and predictable fashion.

USE OF ADENOVIRAL VECTORS FOR CANCER GENE THERAPY

One of the major advantages of adenoviral vectors is that, for a wide variety of cell types, they provide more efficient gene transfer compared with other gene delivery approaches. This is especially true for *in vivo* gene transfer. Recombinant adenoviruses can transfer genes into both proliferating and quiescent cells. One limitation of adenovirus-mediated gene expression is that it is transient, ranging from two weeks to a few months, largely because recombinant adenoviruses are replication-deficient and do not integrate into the host genome. Thus, adenovirus vectors may not be suitable for long-term correction of chronic disorders but should be adequate for therapeutic strategies that require

high and transient gene expression, as evidenced by the increasing numbers of adenovirus vector-based clinical trials submitted to the NIH over the past few years.

Use of Adenoviral Vectors for the Delivery of Toxic or Therapeutic Genes

The ultimate objective of cancer therapy is the selective destruction of tumor tissues and cells. This goal may be accomplished by a variety of means and on a transient basis, followed by systemic administration of adenoviral vectors carrying the therapeutic or toxic genes (Roth and Cristiano, 1997). In the majority of cancer gene therapy trials, adenoviral vectors have been administered *in vivo*, and have been used to transfer drug-sensitive genes (such as the herpes virus thymidine kinase), immuno-modulators (such as IL-2, IL-12, FasL), melanoma tumor antigens (such as MART-1), or tumor suppressor genes (such as p53) (Roth and Cristiano, 1997; Rubinchik *et al*., 2000; Morelli *et al*., 1999; Molnar-Kimber *et al*., 1998; Sterman *et al*., 1998; Motoi *et al*., 2000; Herman *et al*., 1999). To date, many tumor types have been treated with adenoviral based gene transfer. These include melanoma, prostate cancer, mesothelioma, pancreatic cancer, metastatic liver cancer originated from colorectal cancer, lung cancer, neuroblastoma, glioblastoma, overian cancer, and squamous cell carcinoma of the head and neck (Roth and Cristiano, 1997; Molnar-Kimber *et al*., 1998; Sterman *et al*., 1998; Herman *et al*., 1999; Sterman *et al*., 1998; Schuler *et al*., 1998; Habib *et al*., 1999). To exploit FasL/Fas-mediated apoptosis in cancer cells, the Lowenstein group has also created an adenoviral vector expressing FasL (Morelli *et al*., 1999). In order to abolish peripheral liver toxicity, they have packaged the FasL gene in an adenovirus with expression driven by cell type-specific promoters. Similarly, Dong and colleagues have also utilized a tetracycline-inducible system to control the expression of a replication deficient adenoviral vector that delivers a Fas ligand (FasL) protein (Rubinchik *et al*., 2000). FasL induces apoptosis when bound to the Fas receptor and holds great potential as a potent chemotherapeutic agent. Thus, the system is a potentially valuable tool for FasL-based cancer gene therapy and for the study of FasL/Fas-mediated apoptosis. Recently, adenoviral vectors have also been used to transfer antiangiogenic genes for cancer therapy. Extensive experimental and clinical data has substantiated the notion that tumor growth is critically dependent on angiogenesis and that vascular endothelial growth factor (VEGF) plays a central role in the process of tumor neovascularization. It has been shown that adenovirus-mediated anti-VEGF therapy can be used for regional control of tumor growth (Crystal, 1999). Further, systemic administration of adenoviral vector expressing secretable endostatin proved to be an effective antiangiogenic gene therapy for cancer in xenograft models (Chen *et al*., 2000; Sauter *et al*., 2000).

Use of Conditionally Replicative Adenoviruses for Cancer Therapy

To exploit the cytotoxicity associated with wild-type adenoviral replication, a number of strategies have been explored to develop conditionally replicative adenoviruses (CRAds) engineered to produce progeny strictly in tumor cells (For comprehensive review of this subject, refer to: Alemany *et al*., 2000). For instance, in order to take advantage of the frequent mutations of the p53 tumor suppressor in human cancer, McCormick and colleagues utilized an E1b-55kDa-deleted adenovirus that would replicate selectively in *p53*-deficient cells (Bischoff *et al*., 1996). The wild-type E1B55k gene product binds *p53* and inactivates it, promoting viral replication. Thus, only cells that have lost *p53* will allow the CRAd replication, although subsequent studies have revealed that only within certain cell lines is the E1B-55kDa-deleted adenovirus's replication correlated with *p53* status. Hernandez-Alcoceba *et al* developed a CRAd for the treatment of breast cancer (Hernandez-Alcoceba *et al*., 2000) . In this vector, the expression of adenoviral E1A and E4 genes was under the control of a promoter containing estrogen-responsive elements. This promoter induced transcriptional activation of the E1A and E4 genes in response to estrogens in cells that expressed the estrogen receptors, and hence this adenoviral vector was able to kill ER+ breast cancer cells as efficiently as wild type adenovirus.

Similar approaches have used alpha-fetoprotein and prostate specific antigen promoters to control E1A expression to treat hepatocellular and prostate carcinomas, respectively (Yu *et al*., 1999; Hallenbeck *et al*., 1999; Rodriguez *et al*., 1997). A novel approach designed by Castro and colleagues created a dual adenoviral vector system that is both cell-type specific and inducible, with future therapeutic potential to treat brain tumors (Smith-Arica *et al*., 2000). In the dual adenoviral vector system, the expression of tetracycline-dependent transcriptional elements is under the promotion of either the astrocyte-specific, glial fibrillary acidic protein, or the neuronal specific enolase promoter. Thus, viral gene expression can be turned off by administering doxycycline, an innocuous tetracycline derivative, and is restricted to certain cell types that posses the transcriptional activators. Finally, to push the potential therapeutic efficacy of conditional replicative adenovirus to the limit, Rogulski *et al* constructed a replication competent adenovirus expressing a cytosine deaminase/herpes thymidine kinase fusion protein that allowed lytic viral therapy in combination with double suicide gene therapy (Rogulski *et al*., 2000). The cytosine deaminase and herpes thymidine kinase convert systemically delivered, innocuous prodrugs 5-fluorocytosine and ganciclovir, respectively, into toxic metabolites. The double suicide gene therapy was shown to have significant antitumor activity and dramatically potentiated the effectiveness of radiation therapy. Importantly, many clinical studies with encouraging signs of efficacy involve the use of adenoviral vectors. One of the more promising studies is to use the wild-type p53 tumor suppressor to induce tumor cell death (Swisher *et al*., 1999). A series of phase II studies is under way to test this vector alone and in combination with chemotherapies for the local management of various cancers.

USE OF ADENOVIRAL VECTORS FOR NON-CANCER GENE THERAPY

Monogenic Diseases

Adenoviral vectors have also played an important role in gene transfer studies directed to several monogenic diseases. For example, the discovery of cystic fibrosis gene, cystic fibrosis transmembrane conductance regulator (CFTR), immediately established the feasibility of gene therapy for this disease. Adenoviral vectors expressing CFTR have been used in phase I clinical studies on the biosafety and efficacy of gene transfer (Zuckerman *et al*., 1999). Detectable gene transfer was observed in harvested bronchial epithelial cells four days after vector instillation and diminished to undetectable levels by day 43. Adenovirus-specific cell-mediated T cells were induced in most subjects, although only mild increases in humoral immune response were observed. These results demonstrated that gene transfer to the lower respiratory tract epithelium can be achieved in humans with adenoviral vectors but gene transduction is of short duration in the native airway. Further, because of host immune responses, additional administrations may not lead to repetitive expression (Harvey *et al*., 1999). Muscular dystrophy (MD) represents another candidate disease for gene therapy. This therapy is more challenging, however, because of the large amount of muscle tissue in the body, the large size of defective genes in different muscular dystrophies, and possibility of host immune response against the therapeutic gene. Overcoming these challenges requires the development of suitable gene transfer vectors (Douglas and curiel, 1997; Wakefield *et al*., 2000; Gilbert *et al*., 1999; Yuasa, 1998). Encouraging progress has been made to improve adenoviral vectors by modifying the tropism for gene transfer to muscle cells and increasing transgene accommodation capacity. For instance, recently developed gutless adenoviral vectors can deliver full-length dystrophin cDNA to muscle tissue (Clemens *et al*., 1996; Floyd *et al*., 1998; Haecker *et al*., 1996). In combination with adeno-associated virus vectors, adenovirus may provide a promising delivery vehicle for MD gene therapy. Finally, hemophilia diseases represent another group of genetic disorders for which gene therapy may provide an eventual cure. In fact, many studies have already been conducted using improved adenoviral vectors to express therapeutic genes, such as human coagulation factors VIII and IX (Zhang *et al*., 1999; Bristol *et al*., 2000; Balague *et al*., 2000; Roy *et al*., 1999; Lipshutz *et*

al., 1999; Connelly *et a*l., 1999). Sustained transgene expression and correction of hemophiliac phenotypes have been observed in animal models.

Genetic and Metabolic Liver Diseases

Because adenoviral vectors have been shown to mediate efficient gene transfer to hepatocytes (Ferry and Heard, 1998), adenoviral directed gene delivery has been pursued as potential therapies for a wide variety of genetic and metabolic liver disorders, such as lysosomal storage diseases, glycogen storage diseases, phenylketonuria, and Tay-Sachs disease (Eto and Ohashi, 2000; Stein *et al*., 1999; Ziegler *et al*., 1999; Zingone *et al*., 2000; Amalfitano *et al*., 1999; Nagasaki *et al*., 1999; Guidotti *et al*., 1999). Interestingly, expression of the liver-specific α2 interferon, delivered by adenoviral vectors, has led to effective protection of the animals from induced hepatitis (Aurisicchio *et al*., 2000). More recently, an adenoviral vector expressing the essential telomerase RNA gene has been shown to restore telomerase activity, alleviate cirrhotic pathology, and improve liver functions in an experimental liver cirrhosis model (Rudolph *et al*., 2000). Although adenoviral vectors mediated high levels of transgene expression in liver cells, it remains a major challenge to maintain prolonged gene expression and to circumvent immune response against viral vectors.

Neurodegenerative Diseases

Recently, the efficacy of adenoviral vectors has been demonstrated in several models of neurodegenerative diseases including Parkinson's disease (PD) and motor neuron diseases (Barkats *et al*., 1998; Smith, 1998). In rat PD models, adenoviral vectors expressing either tyrosine hydroxylase, superoxide dismutase or glial-derived neurotrophic factor improved the survival and functional efficacy of dopaminergic cells (*Corti et al*., 1999). In a search for therapeutic agents to treat another neurodegenerative disease, Huntington's Disease(HD), Bemelmans *et al* delivered brain-derived neurotrophic (BDNF) factor via adenoviral vector to rat models (Bemelmans *et al*., 1999). HD is a genetic disorder leading to the degeneration of striatal GABA-ergic output neurons and BDNF has been shown to be beneficial for striatal neuron survival. Their study indicated that transfer of the BDNF gene was of therapeutic value for Hunting-ton's disease. Similarly, intramuscular injection of an adenoviral vector encoding for neurotrophin-3 demonstrated substantial therapeutic effects in a mouse model of motor neuron degenerative disease. Interestingly, the immunosuppressant FK506 was shown to prolong adenovirus-mediated transgene expression in brain cells of experimental animals (Durham *et al*., 1997).

Cardiovascular Diseases and Tissue Regeneration

At least in animal models, adenoviral vectors have also been shown to effectively transduce therapeutic genes for several cardiovascular diseases, such as atherosclerosis, cerebral ischemia, familial hypercholesterolemia, hypertension, and cardiac arrhythmias (Gerrard and Collen, 1997; Kevin *et al*., 2000; Papadopoulos *et al*., 2000; O'Brient and Simari, 2000; Tangirala *et al*., 1999; Stein *et al*., 2000; Ponder, 1999). As with other diseases, adenoviral gene therapy needs improved long-term expression and regulation in response to physiological changes, before the systems can be implemented as therapies in humans. One innovative approach to adenoviral gene therapy was developed by the Marban group, who used adenoviral vectors to treat cardiac arrhythmias by modifying focal electrical conduction in the heart (Kevin *et al*., 2000). Their approach utilized a porcine heart model and delivered adenovirus encoding the Galphai2 subunit to the atrioventricular node. When expressed in excess, Galphai2 acts like a beta-adrenergic antagonist. They found that with gene overexpression, the baseline atrioventricular conduction was suppressed and the heart rate was slowed during atrial fibrillation without producing complete heart block. Recent studies have demonstrated a highly promising utility of adenoviral vectors in regenerative medicine and tissue engineering. Particularly, several groups have demonstrated that adenoviral vectors expressing BMP2, BMP7,

BMP9, or BMP12 can induce significant bone formation and fracture healing in animal models (Franceschi *et al*., 2000; Helm *et al*., 2000; Lou *et al*., 1999; Lou *et al*., 1999; Krebsbach *et al*., 2000; Riew *et al*., 1998; Musgrave *et al*., 1999; Gonda *et al*., 2000; Alden *et al*., 1999; Alden *et al*., 1999; Baltzer *et al*., 2000; Okubo *et al*., 2000). It is conceivable that adenoviral vectors can be used to efficiently transduce certain growth factors that may promote bone and cartilage regeneration as well as ligament repair and wound healing.

LIMITATIONS AND IMPROVEMENTS OF ADENOVIRAL VECTORS

Although recombinant adenoviral vectors have become increasingly popular gene delivery vehicles, there are two major limitations that could hamper their eventual use in human gene therapy. First, the adenoviral vectors usually mediate a short-term gene expression; and second, adenoviral vectors tend to elicit strong immune and inflammatory responses *in vivo*. A single large dose of adenovirus can efficiently provoke production of neutralizing antibodies directed to the viral particle, which in turn would preclude or reduce the efficiency of repeated systemic administration. It has been reported that about 55% of adult humans have a low titer of neutralizing antibodies against adenovirus (Chirmule *et al*., 1999), but it is not yet apparent whether these pre-existing antibodies will significantly interfere with gene transfer upon the systemic administration. Moreover, it appears that even at high titer the neutralizing antibodies in blood would not reduce gene transfer by repeated intra-tumor injections (Swisher *et al*., 1999). Nevertheless, consi-derable improvements have been made on the adenoviral vector technology for the past few years.

Several approaches have been explored to circumvent the immunogenicity of adenoviral vectors by changing the vector designs. For instance, a new generation of adenoviral vectors has been constructed with deletion of E1, E2 and E4 genes in order to avoid expression of immunogenic viral proteins in host cells (Ferry and Heard, 1998). Alternatively, constitutive expre-ssion of E3 gp19K protein in E1-deleted vector has provided encouraging results with more stable transgene expression in the liver and lung of animal models (Bruder *et al*., 1997; Ilan *et al*., 1997). The function of gp19K is to inhibit the transport of major histocompatibility complex class I molecules to cell surface, leading to the impairment of antigen-presenting cells' function and reduced clearance of adenoviral infected cells by CTL immune responses (Lee *et al*., 1995; Beier *et al*., 1994). The recently developed gutless adenoviral vectors, which have most or all adenoviral genes deleted, have shown significantly reduced immunogenicity and prolonged expression of homologous transgenes in mice (Schiedner *et al*., 1998; Morsy *et al*., Chen *et al*., 1997). Interestingly, a conceptually different approach was taken to excise significant portions of viral genes from vector backbone, in which the complete E2 region was flanked with loxP sites and removed with Cre recombinase (Lieber *et al*., 1996). The resulting gutless virus mini-chromosome can be further stabilized with E2B-encoded preterminal protein (Lieber *et al*., 1996; Lieber *et al*., 1997). Although low levels of contaminating helper virus presented, gutless vectors do not elicit a strong cellular immune response, while they do induce a humoral response against the viral caspid components at the time of injection.

Many approaches are being developed to control host immune responses at the time of infection. One of them is to transiently block cell adhesion and co-stimulatory molecules, such as CD40 ligand, in order to prevent both cytotoxic response and production of virus-specific neutra-lizing antibodies (Yang *et al*., 1996). Immuno-modulating cyto-kines, such as IL-10 and IL-12, were also used to disrupt the balanced Th activation towards either Th1 (cytotixic) or Th2 (humoral) subset, thereby reducing antibody production and cellular immune response, respectively (Yang *et al*., 1995; Qin *et al*., 1997). Because TNFα has been shown to play a key role in adenovirus-induced immune response, inhibition of this pathway may offer a particularly promising prospect in overcoming host cell immune responses. Indeed, systemic adenovirus injection in TNFα null animals was associated

with a weak acute phase inflammatory reaction, reduced infiltration within the liver, a severely impaired T-cell proliferative response to both adenoviral and transgene proteins, and a significantly reduced level of anti-adenovirus antibodies (Elkon *et al*., 1997; Benihoud *et al*., 1998). Consequently, two strategies are being developed to antagonize TNFα pathway by either bolus injection of soluble TNF receptor or overexpre-ssion of E3B-coded antagonists (Ilan *et al*., 1997; Zhang *et al*., 1998). However, the *in vivo* benefits remain to be seen.

Manipulation of virus capsid components by genetic engineering may provide another alternative to circumvent pre-existing humoral response to the commonly used adenovirus serotype 5. In fact, vector capsids displaying chimeric Ad5/Ad12 hexon monomers were shown to overcome neutralizing antibodies in C57BL/7 mice primed with Ad5 (Roy *et al*., 1998). Interestingly, such chimeric capsid may also change the binding affinity to host cells. For instance, chimeric capsid Ad5/Ad7 exhibited an enhanced binding affinity for human lung epithelial cells but significantly diminished efficiency for liver-directed gene transfer (Gall *et al*., 1996; Miyazawa *et al*., 1999). An adenovirus type 2 chimeric vector expressing the fiber gene from adenovirus type 17 has been shown to efficiently transduce human airway epithelium contingent upon αVβ5 integrin expression (Zabner *et al*., 1999; Goldman and Wilson, 1995). Furthermore, the insertion of an Arg-Gly-Asp (RGD) motif into the fiber gene of an adenoviral vector has generated a chimeric vector with expanded cell tropism and enhanced transduction efficiency in primary tumor cells, while the in-frame fusion of a polylysine moiety to the fiber protein remarkably improved adenoviral trans-duction of muscle cells (Bouri *et al*., 1999; Dmitriev *et al*., 1998). Interestingly, a chemical cross-linking of polye-thylene glycol (PEG)ylation of adenoviral vectors has been shown to effectively protect the viral particles from neutralizing antibodies both *in vitro* and *in vivo* (O'Riordan *et al*., 1999).

While adenoviral vectors can efficiently transfer genes into a broad spectrum of cell types, this wide tropism also represents an apparent drawback when gene delivery to a specific tissue is needed. For the past few years, several strategies have been developed to address this issue. Two basic requirements are necessary to create a targeted adenoviral vector: interaction of adenovirus with its native receptors must be interrupted and tissue-specific ligands must be presented on the viral capsids. Earlier attempts to control adenovirus tropism usually relied on the use of bispecific binding molecules to simultaneously block native receptor binding and redirect virus binding to a tissue-specific receptor. For example, the Fab fragment of a neutralizing monoclonal antibody against the fiber protein conjugated to folate was shown to redirect adenoviral infection to cells expressing folate receptors (Douglas *et al*., 1999). Similarly, a bispecific antibody against both the fiber protein and the epidermal growth factor receptor (EGFR) was able to abrogate native adenovirus binding and redirect viral infection to human glioma cells via EGFR (Miller *et al*., 1998). Alternatively, redirected adenoviral tropism can also be achieved by modifying the fiber protein. It has been reported that viral vectors containing chimeric fiber coat proteins with peptide ligands allowed specific binding to the heparan sulfate and integrin receptors (Wickham *et al*., 1997). More recently, a novel strategy for cell type-specific gene delivery has been developed by modifying adenoviral vectors with biologically selected peptides (O'Riordan *et al*., 1999; Romanczuk *et al*., 1999). Specifically, novel peptide ligands that specifically bind to airway epithelial cells were first isolated by biopanning the cells against a phage display library, and then the peptide with the most effective binding was coupled to the surface of an adenoviral vector using bifunctional PEG molecules. This chemically and biologically modified adenoviral vector was able to efficiently transfer genes into airway epithelial cells. This strategy maybe particularly interesting because coupling of PEG to the surface of adenovirus also protects it from neutralizing antibodies.

Long-term expression of transgenes is desirable for replacement gene therapy. Several strategies

have been developed to address the drawback of adenovirus-mediated transient gene expression. For example, a chimeric adenoviral-retroviral vector has been constructed in order to maintain transgenes within actively dividing cells (Bilbao *et al*., 1997; Feng *et al*., 1997; Duisit *et al*., 1999). This chimeric virus was shown to infect cells and produce recombinant retroviruses that can infect surrounding cells and integrate into host chromosome. Similarly, a hybrid adenoviral/ adeno-associated virus (AAV) was engineered and shown to integrate the transgene at a specific locus of human chromosome 19 (Lieber *et al*., 1999). The major difference between the two types of chimeric viruses is that AdV/AAV vector may also maintain efficient and lasting transgene expression in nondividing cells. Others have also explored the possibility of long-term expression mediated by the EBNA1/OriP episomal replication system derived from Epstein-Barr virus (Benihoud *et al*., 1999).

Finally, the transgene expression mediated by adenoviral vectors may require fine-tuned regulation for some, if not all, therapeutic applications. Currently, expression of most transgenes is driven by ubiquitous promoters of viral origin, such as the immediate-early promoter from human cytomegalovirus (CMV) and the Rous sarcoma virus long terminal repeat (LTR). Although these promoters provide high levels of transgene expression, it is not always desirable and specific enough for a wide variety of therapeutic applications. In this respect, endogenous promoters have been used to restrict transfer gene expression to particular cell types at a physiologically relevant level. For instance, tissue-specific transgene expression was achieved when a transactivator was coupled with a liver-specific promoter (Aurisicchio *et al*., 2000; Pastore *et al*., 1999). Similarly heart-specific gene expression was accomplished using the ventricle-specific cardiac myosin light chain 2 promoter in adenoviral vectors. Other regulatable gene expression systems, such as tetracycline and RU486-inducible systems, have also been incorporated into the adenoviral backbone and regulated transgene expression has been demonstrated (Molin *et al*., 1998; Corti *et al*., 1999; Harding *et al*., 1998; burcin *et al*., 1999). Moreover, in combination with other genetically manipulated systems, adenoviral vectors can also be used to activate or inactivate specific genes *in vivo*. For example, adenoviral vector expressing Cre recombinase can be used in transgenic models in which loxP sites have been incorporated into the genome to accomplish targeted gene activation or inactivation (Wang *et al*., 1996; Agah *et al*., 1997; Anton and Graham, 1995; Shibata *et al*., 1997).

CONCLUSIONS

With the completion of the human genome project and the emergence of functional genomics, the future of human gene therapy and molecular medicine is brighter than ever. This will ultimately change the practice of medicine and the way that drugs are discovered. However, in the near future, more basic and translational research has to be devoted to the development of delivery vectors and the improvement of gene transfer efficacy. Ten years of gene therapy research has taught us many important lessons. One of them is that one vector does not necessarily fit all. It is conceivable that any effective gene therapy should be tailored for a specific disease in terms of gene transfer. Adenoviral vector offers a great promise in this respect. Decades of basic research on adenovirus biology will certainly facilitate the development and refinement of such gene transfer systems.

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