Prospects for Virus-Based Gene Therapy for Cystic Fibrosis

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Gene therapy for cystic fibrosis (CF) could potentially be accomplished with one of several recombinant virus vectors, including a murine retrovirus (MMuLV), adenovirus, or adeno-associated virus (AAV). All these vectors take advantage of their respective viruses' mechanisms for delivery of viral DNA to cells, evasion of lyosomal degradation, and optimization of the levels and duration of expression of viral (or vector) DNA. Each has its own unique life cycle, however. The differences among these viruses result in certain advantages and disadvantages, such as the requirement of retroviruses for active cell division, and the potential pathogenic effects from expression of certain adenovirus genes present in adenovectors. While no single vector may be optimal for CF gene therapy in humans, new techniques, such as receptor-mediated gene transfer, seek to take advantage of the desirable properties of one or more of the virus-based systems while avoiding certain potential hazards.

KEY WORDS: Gene therapy; cystic fibrosis (CF); retrovirus; adenovirus; adeno-associated virus (AAV).

INTRODUCTION

The median life expectancy for patients with cystic fibrosis (CF) increased from less than 10 years in 1960 to more than 27 years[1,2] in 1989 due solely to improvements in antibiotic therapy, nutritional support, and rigorous center-based clinical follow-up. The discovery[3-5] of the CF gene in 1989 promised to accelerate the pace of therapeutic gains for CF patients even further. Substantial advances have been made in the basic knowledge of the CF defect and in the development of technologies potentially relevant to CF gene therapy. It remains to be seen whether these advances will lead to any rapid breakthrough in the outcome of a disease which has already become more of a chronic medical condition than a fatal childhood illness.

VIRUS-MEDIATED *IN VIVO* GENE TRANSFER

The principle underlying somatic gene therapy for recessive genetic diseases like CF is based on the observation that heterozygous individuals are phenotypically normal. This implies that expression from the normal CF gene is not interfered with by the mutant allele, and presumably a normal gene copy inserted into a homozygous mutant cell could likewise function. This type of gene therapy is known as *gene augmentation*.[6] Since the gene is to be inserted at a site distant from the original locus, the transcription promoter and other desired regulatory elements must be provided with it. In the case of CF, the best strategy may be to express as much CFTR as possible in as many cells as possible, particularly since there appears to be no deleterious effect from CFTR overexpression in transgenic animals.[7]

Although there are many methods for accomplishing gene transfer *ex vivo*,[8] only transducing virus vectors, liposomes,[9,10] and the receptor-mediated gene transfer[11] methods are currently feasible *in vivo*. The principal viral vectors potentially useful for CF gene therapy include retroviruses,[12] e.g., the Moloney murine leukemia virus (MMuLV), adenoviruses,[13,14] and adeno-associated virus (AAV), a nonpathogenic human parvovirus.[15,16] In each case, vectors have been constructed in which the CF gene sequences

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Table I. Advantages and Disadvantages of Viral Vectors Potentially Useful for CF Gene Therapy

MMuLV
    Advantages
        High-frequency integration
        Wild-type virus is not pathogenic
        Clinical experience in human gene therapy trials
    Disadvantages
        Requires actively dividing cells
        Relatively low titers
        Some risk of insertional mutagenesis

Adenovirus
    Advantages
        High levels of expression
        Very high titers
        Natural respiratory and g.i. tract tropism
    Disadvantages
        Wild-type virus is pathogenic
        Vectors contain many virial genes
        Risk of immunologic reactions with repeated exposures

Adeno-associated virus
    Advantages
        Stable, site-specific, high-frequency integration
        Wild-type virus is not pathogenic
        Relatively high titers
        Natural respiratory and g.i. tract tropism
    Disadvantages
        Lack of packaging cell line
        Risk of immunologic reactions with repeated exposures

replace portions of the viral sequence to form a hybrid sequence which can be packaged into the virus particle. The particles will then deliver the normal CF gene copy with high efficiency into the target cells, utilizing the natural viral infection mechanisms for cell surface attachment and avoidance of lysosomal degradation. Although these general features are common to all three type of vectors, there are substantial differences among them, due to differences in the biological characteristics of the viruses on which they are based. These differences result in certain advantages and disadvantages regarding their potential usefulness in CF gene therapy,[2] some of which are listed in Table I.

RETROVIRUSES

Retroviruses, such as MMuLV, have a single-stranded RNA genome which is converted to DNA by reverse transcriptase carried in the virus particles.[17] If the infected cell is actively dividing, the DNA provirus is then randomly integrated into the host cell genome and viral genes are expressed, allowing for the transcription of new RNA copies and the formation of

new particles. These virions bud from the producer cell membrane and travel to infect other cells and perpetuate the life cycle. The genome consists of viral genes, *gag-pol* and *env*, flanked by two long terminal repeat (LTR) sequences. Just inside the left-hand LTR is the packaging signal, *psi*, which targets viral RNA to be encapsidated (or packaged) into virus particles.

Recombinant MMuLV vectors are constructed by replacing the *gag-pol* and *env* genes with another gene of interest, such as the CF gene. Packaging cell lines have been designed to supply the *gag-pol* and *env* genes from a different DNA strand with a mutated *psi* packaging sequence.[17-20] The viral genes provide the proteins needed for the production of packaged virions, but only the recombinant CF-retrovector RNA (psi^+) is encapsidated. Increasing titers can be produced by reinfecting vector into a second packaging cell line. Unfortunately, retrovector preparations generally are produced at relatively low titers, 10^5–10^7/ml.

There is a great deal of experience with retrovirus vectors both *in vitro* and *in vivo*, including small trials in humans. In tissue culture, MMuLV vectors have been used to express a wide variety of genes, including adenosine deaminase,[21] low-density lipoprotein receptor,[22] beta-globin,[23,24] hypoxanthine-guanine phosphoribosyl transferase,[25,26] and many others,[27-29] including CFTR.[12] The first successful complementation of the CF defect involved a retrovector used to transduce a CF pancreatic adenocarcinoma cell line (CFPAC1). After complementation, these cells demonstrated restoration of cAMP-regulated chloride conductance, characteristic of normal CFTR function.[12] Early human trials with other retrovirus vectors have involved *ex vivo* transduction of lymphocytes (either from tumors or peripheral blood) which can then be reinfused and used for cell-tracking[30] or therapeutic purposes, as in children with adenosine-deaminase (ADA) deficiency[31] resulting in the severe combined immune deficiency syndrome.

There are significant doubts as to whether an *ex vivo* approach will be feasible for the airway epithelium which seems to be the key to the pathophysiology of lung disease in CF patients. A tracheal xenograft model[32] has been used to approach this problem. Primary human airway epithelial cells grown in culture can be transduced with retrovirus vectors and then used to repopulate a denuded tracheal collagen matrix which will then survive in a nude mouse recipient. The transduced airway cells will then stably

express the recombinant gene. Applying this approach to human recipients remains problematic. Another approach might entail using *in vivo* infection with the vector, but only a small percentage of cells at the airway surface are actively dividing. In culture, less than 10% of cells in a monolayer need to be transduced in order to see restored cAMP-responsive chloride conductance,[33] but these cells are coupled by gap junctions which disappear from human airways during the canalicular stage of lung development.[34] Additional disadvantages of retrovectors include the low titers and the risk of insertional mutagenesis mentioned earlier.

ADENOVIRUSES

Adenoviruses are naked icosahedral double-stranded DNA viruses, which commonly infect the respiratory and gastrointestinal tracts of humans and other primates.[35] The genome consists of approximately 35 kb of DNA, including late genes (structural genes transcribed after DNA replication), early genes (whose transcription is required for DNA replication), and the immediate early gene E1A which is a multi-functional transcription regulator required to activate expression from the other genes.[36] In a productive life cycle, adenovirus binds to a cell surface receptor and enters the cell, lysing its endosome before lysosomal degradation can occur. If E1A is active, transcription of early genes then occurs in the nucleus, followed by DNA replication, late gene expression, virus particle assembly, and cell lysis. Without E1A activity, the other genes are expressed at very low levels, so very little replication can occur. Of note, one early gene, E3, is not required for viral replication but helps to evade the immune response by suppressing HLA antigen presentation.

Vectors are made by deleting the E1A region (and sometimes E3) and substituting the foreign sequence, such as CFTR.[13,14] These vectors can be packaged in the 293-1 cell line which constitutively expresses E1A. Vector particles are then produced which are E1A⁻, and so should be able to enter target cells, but express only the recombinant gene which is transcribed from a promoter which is not E1A dependent.

Adenovectors have been shown to provide high levels of expression in the airway epithelium *in vivo* as well as *in vitro*. Beta-galactosidase, alpha-1-antitrypsin,[13] and CFTR[14] have all been expressed in the cotton rat lung. *In vivo* hepatocyte expression is also

feasible,[37] although the levels of expression appear to be lower. Although recombinant adenovirus vaccines have been used in humans, some questions remain regarding the safety of human gene therapy with adenovectors. Although the initial levels of expression are excellent, there appears to be no way to target progenitor cells in the airway, and stable integration may not occur at high frequency. If repeated administrations are needed, an immune response would likely occur. In the respiratory tract, this could cause bronchospasm or other adverse reactions. It is also possible that the vectors could express adenoviral genes and/or replicate, either with the help of any natural coincident adenovirus infection or in any respiratory tract cells expressing E1A or E1A-like activity. This could be undesirable since CF patients often have pulmonary exacerbations triggered by otherwise mild respiratory tract infections. Despite these concerns, there is a growing body of evidence from animal experiments which indicates that adeno-CF vectors will be relatively safe and efficacious in humans, and clinical trials are currently being planned.

ADENO-ASSOCIATED VIRUS (AAV)

AAV is a small (20 nm) icosahedral single-stranded DNA virus of the parvovirus family.[38] It was initially isolated as a tissue culture contaminant, and subsequently found as a human isolate coinfecting the respiratory and gastrointestinal tracts of young children during an adenovirus outbreak.[39] It is nonpathogenic and does not appear to alter the clinical course of concurrent adenovirus infection. AAV is naturally defective for replication, so that it requires coinfection with a helper adenovirus or herpesvirus for replication in a productive life cycle. In the absence of helper virus infection, AAV undergoes high-frequency stable DNA integration, usually into a specific site on chromosome 19.[40-42] Integrated AAV-DNA can be "rescued," i.e., excised and replicated, if helper virus infection occurs at a later time.

The 4.7-kb AAV genome consists of two genes, *rep* and *cap*, flanked by the inverted terminal repeats (ITRs) which serve as origins of DNA replication and packaging signals,[38] and also influence gene expression.[16] Alternate splicing and the use of an internal translation start site allow for the production of the three capsid proteins (VP1, VP2, and VP3) from the one *cap* gene promoter (p40). By use of two different promoters (p5 and p19) and alternate splicing, a total

of four Rep proteins are produced which function as transcription regulators and to allow for terminal resolution of replicating AAV genomes, a vital step in producing progeny. Rep also suppresses other viruses, such as HIV1,[43] and inhibits carcinogenesis in a variety of animal and tissue culture models.[44,45]

AAV vectors take advantage of a natural tropism for the respiratory and gastrointestinal tracts and its mechanism for stable DNA integration. As with other vectors, a gene of interest, such as CFTR, is substituted for the viral genes, rep and cap, between the two ITRs. Since there are no stable helper-free packaging cell lines for AAV, packaging is accomplished by a cotransfection technique,[46] in which adenovirus-infected cells receive both the AAV-CF vector and a packaging plasmid which contains only the rep and cap sequences without the ITRs. As with retrovectors, only the appropriate vector sequence flanked by the ITRs will be encapsidated. Vector particles are released by lysing the cells and can be concentrated in a CsCl gradient to titers greater than 10^{10}/ml.[16] AAV vectors prepared in this way have been used for transfer of marker genes, such as neo and cat,[16,46–48] and for transfer of the gamma-globin gene to erythroid precursor cells.[49]

Because of the large size of the CFTR cDNA coding sequence (4.5 kb) relative to AAV, the selection of an optimal small promoter is of critical importance. Our group initially tested AAV-p5 promoter expression in a CF bronchial epithelial cell line, IB3-1, since the p5 promoter along with the left-hand ITR comprises a convenient 263-bp cassette.[16] The p5 promoter was 5 to 10 times more active for transient and stable expression than the SV40 promoter, and the ITR was shown to increase gene expression, suggesting an enhancer effect. Packaged AAV p5-neo vectors were able to transduce up to 75% of cells to geneticin resistance. AAV p5-CF vectors were constructed but were somewhat larger than wild-type AAV. A serendipitous observation was made that the ITR itself has transcription promoter activity,[50] probably related to its containing a consensus initiator sequence.[51] A 145-bp ITR element was isolated which was sufficient for replication origin, packaging signal, and promoter activity. This has allowed for the production of AAV-CF vectors which are smaller and able to be packaged at high titers.

Several different versions of the AAV-CF vector have been used for in vitro complementation of the CF defect in the IB3-1 cell line. These vectors were shown to be functional by transfection or packaged vector transduction in the absence of selection,[50,52] AAV-CF complemented cells show restoration of cAMP-responsive chloride conductance. Patch-clamp experiments show the appearance of the 10-ps linear chloride conductance characteristic of recombinant CFTR expression and a return to cAMP-responsiveness by the outwardly rectifying chloride channel, which may indicate an important regulatory role of CFTR in these differentiated epithelial cells.[15] AAV-CF vector transduction has also been performed in primary CF nasal polyp cells, and in vivo experiments are underway with AAV-CAT and AAV-CF vectors in rodent and rabbit models.

AAV vectors have theoretical advantages over retrovectors in that integration is relatively site-specific, providing a lower risk of insertional mutagenesis. Also the titers are higher, nearly approaching those achievable with adenovectors. The principal advantages over adenovectors are the absence of other viral genes and the nonpathogenic nature of wild-type AAV. Adverse immunologic reactions are also possible with AAV, however, and AAV-CF vectors could be mobilized by coincident wild-type AAV and adenovirus infection.

FUTURE DIRECTIONS

The recent development of a transgenic CF knock-out mouse provides the first true animal model of CF[53] and is certain to play a major role in gene therapy experiments for CF in the near future. These mice demonstrate a phenotype equivalent to the pancreatic insufficiency and meconium ileus seen in newborns with severe CF disease manifestations. There are also pathological[53] and physiological[54] changes in the respiratory epithelium. It will be the task of any new viral or nonviral gene transfer system to prove its efficacy in this animal model.

Viral vectors have provided efficient vehicles for tissue culture and in vivo CF gene transfer experiments, and one or more will likely prove efficacious in the CF$^-$ mice and subsequently be used in human trials. Each does have disadvantages, however, and it may be that no single one will ultimately provide an optimal agent for therapy. The ability to conjugate certain DNA elements with protein moieties may provide a means to combine advantageous aspects of each of these vectors into a final product which is more efficacious. For example, one might utilize the adenovirus capsid proteins for cell entry and evasion

of lysosomal degradation[11] combined with AAV sequence elements crucial for site-specific DNA integration. These ideas essentially represent modifications of receptor-mediated endocytosis and liposome techniques which to date have demonstrated low levels of expression, but may avoid the potential hazards of using recombinant viruses in patients who are already compromised by their underlying disease.

One additional aspect of gene therapy research is its impact on the general understanding of CF pathophysiology. An example of that is the work of Egan et al.,[15] in which an AAV-CFTR gene transfer experiment resulted in an improved understanding of a regulatory role of CFTR on alternate chloride conductance pathways. These observations raise the possibility of bypassing the regulatory step and using pharmacologic agents which might activate these non-CFTR epithelial chloride channels directly. Basic questions about the pathophysiology of CF also strongly impact on strategies for gene therapy. For example, recent evidence points to a significant role of normal CFTR function in a variety of processes, including regulation of endosomal pH, endosome turnover, and mucin secretion. These findings call into question the model of complementation consisting simply of restoration of cAMP regulation of chloride efflux. Although the model of cellular disease in CF is becoming more complex, it is reasonable to expect that this more detailed understanding will bring us closer to effective therapies. The mutual interdependence of "gene therapy" experiments, basic molecular biology, and cell physiology in CF research continues to raise hopes that clinical benefits may soon be reaped from this growing body of work.

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