

Gene Therapy: The First Two Decades and the Current State-of-the-Art

TERENCE R. FLOTTE*

Department of Pediatrics, University of Massachusetts Medical School, Worcester, Massachusetts

The concept of gene therapy was envisioned soon after the emergence of restriction endonucleases and subcloning of mammalian genes in phage and plasmids. Over the ensuing decades, vectors were developed, including nonviral methods, integrating virus vectors (gammaretrovirus and lentivirus), and non-integrating virus vectors (adenovirus, adeno-associated virus, and herpes simplex virus vectors). Preclinical data demonstrated potential efficacy in a broad range of animal models of human diseases, but clinical efficacy in humans remained elusive in most cases, even after decades of experience in over 1000 trials. Adverse effects from gene therapy have been observed in some cases, often because of viral vectors retaining some of the pathogenic potential of the viruses upon which they are based. Later generation vectors have been developed in which the safety and/or the efficiency of gene transfer has been improved. Most recently this work has involved alterations of vector envelope or capsid proteins either by insertion of ligands to target specific receptors or by directed evolution. The disease targets for gene therapy are multiple, but the most promising data have come from monogenic disorders. As the number of potential targets for gene therapy continues to increase, and a substantial number of trials continue with both the standard and the later generation vector systems, it is hoped that a therapeutic niche for gene therapy will emerge in the coming decades.

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In the late 1970s and early 1980s, the emergence of techniques for subcloning mammalian genes into prokaryotic plasmids and bacteriophages were correctly foreseen as precursors to techniques for human gene therapy. The parallel investigations of the biology of avian and murine onco-retroviruses led to the development of retroviral vectors, which were in common laboratory use by the mid-1980s as a means to transfer genes into mammalian cells. Thus, by the late 1980s the stage was set for the first human trials of gene transfer, which included the first gene-marking study of tumor-infiltrating lymphocytes (TIL) by Dr. Steven Rosenberg's group in 1989 (Culver et al., 1991; Rosenberg et al., 1990) and the first gene therapy study in peripheral blood T lymphocytes of patients with severe combined immune deficiency (SCID) due to adenosine deaminase (ADA) by Dr. R. Michael Blaese's group, which commenced in 1990 (Blaese et al., 1995).

Since that time there has been a remarkable expansion of both the number of human genes directly associated with disease states and the number of vector systems available to express those genes for therapeutic purposes. Gene therapy with recombinant adenovirus (rAd) vectors commenced in April 1993 with a trial in cystic fibrosis (CF) patients (Crystal et al., 1994), and 2 years later the first recombinant adeno-associated virus (rAAV) trial was also initiated in CF patients in November 1995 (Flotte et al., 1996, 2003), with trials in hemophilia B patients commencing shortly thereafter (Kay et al., 2000a). Subsequently, lentivirus and recombinant herpesvirus vectors have also entered the clinical arena (MacGregor, 2001; Glorioso et al., 2003; Bank et al., 2005; Levine et al., 2006; Mata et al., 2006). Each of the latter four vector systems is capable of gene transfer and expression in vivo in non-dividing cells. A variety of non-viral gene transfer methods, including cationic liposomes, DNA conjugates, and naked DNA injection, also came into use in gene therapy and recombinant vaccine trials in the 1990s. Each of these vector systems has its own advantages and disadvantages (Table 1).

Limitations of First-Generation Vectors

As data from this wide variety of trials with each of these gene transfer systems have been generated, a number of notable difficulties have come to the fore. First-generation recombinant Ad vector triggered a dose-dependent induction of both innate

and adaptive immune responses, which have not only limited the duration of expression, but have also resulted in severe inflammatory toxicity in some patients, causing at least one death in a patient with partial ornithine transcarbamylase (OTC) deficiency (Raper et al., 2003). The original onco-retrovirus (now termed gamma-retrovirus) vectors, when used on bone marrow stem cells from patients with X-linked SCID have caused leukemia-like syndromes, apparently due to insertion activation of the LMO2 oncogene (Hacein-Bey-Abina et al., 2003). Each of these severe toxicities is reminiscent of the pathology associated with the parental virus on which the vector-system is based. Wild-type Ad triggers robust innate and acquired immune responses in humans. In those instances where systemic Ad viremia occurs, such as in neonates, it can cause a fatal systemic inflammatory response syndrome (SIRS) resembling that seen in the Ad-OTC trial. Likewise, the retroviral vectors implicated in the X-SCID trial toxicity are derived from a virus known as Moloney murine leukemia virus. Thus, in hindsight the nature of these toxicities might have been anticipated.

While not associated with such severe toxicities, other vector systems have not been overwhelmingly successful, in terms of their efficacy in clinical gene therapy trials. The in vivo efficiency of non-viral systems has been low and transient. Although it was initially thought that non-viral vectors would be free of inflammatory toxicity, aerosol administration has been associated with an influenza-like syndrome (Ruiz et al., 2001). First-generation rAAV2 vectors appear to elicit very little innate response and demonstrate a longer duration of expression in a wide range of tissues, including muscle, brain,

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*Correspondence to: Terence R. Flotte, Department of Pediatrics, University of Massachusetts Medical School, Worcester, MA.
E-mail: Terry.Flotte@umassmed.edu

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TABLE 1. Characteristics of vector systems currently in human trials

Vector	Advantages	Disadvantages	Number of US clinical trials	Examples of disease applications
Gamma-retrovirus	High efficiency integration	Insertional mutagenesis with secondary malignancy	>600	ADA-SCID, X-SCID, CGD, familial hyperlipidemia, tumor vaccine
Lentivirus	Stable gene transfer Non-dividing cells	Requires cell division Insertional mutagenesis with secondary malignancy	6	HIV, MPS-type VII
Adenovirus	Stable gene transfer Non-dividing cells	Risk of replication competent HIV Innate immunity	194	CF, ornithine transcarbamylase deficiency, mesothelioma, colon cancer
Adeno-associated virus	High efficiency Non-dividing cells	Transient gene transfer Small packaging limit	32	CF, hemophilia B, Leber congenital amaurosis, Canavan disease, AAT deficiency
Herpesvirus	Minimal innate response Stable gene transfer Non-dividing cells	Adaptive immune response to capsid Potential for innate and adaptive immunity	13	Brain tumors, colon cancer, intractable pain
Non-viral	High efficiency Non-dividing cells Large packaging capacity	Innate immunity Transient gene transfer	156	CF, DNA vaccines, metastatic cancer

Note that the number of US clinical trials is based on all trials listed by the NIH Office of Biotechnology Activities (OBA) GEMCRIS website. Some of those trials may not yet have been initiated, but all have been IRB-approved and reviewed by OBA and/or the Recombinant DNA Advisory Committee (RAC).

retina, liver, and lung (Flotte et al., 1993, 2003, 2005; Conrad et al., 1996; Kessler et al., 1996; Fisher et al., 1997; Snyder et al., 1997; Song et al., 1998, 2001; Wagner et al., 1998, 1999; Xiao et al., 1998; Kay et al., 2000b; Acland et al., 2001; Manno et al., 2003). However, difficulties with adaptive immune responses to the retained viral capsid proteins represent an important limitation in some contexts (Manno et al., 2006). While neither one of these host responses appears to have resulted in clinically important toxicity, they may be limiting the clinical efficacy of gene therapy.

Ironically, the only indications of clinically meaningful efficacy with gene therapy have been with the onco-retrovirus vectors for X-SCID (Thrasher et al., 2006) and chronic granulomatous disease (CGD; Ott et al., 2006) and with a first-generation rAd vector expressing p53 (Swisher et al., 2003; Peng, 2005). Since both of these systems are associated with important limiting toxicities, the search for new safe and effective vectors continues.

Attempts to Improve Vector Design

Modifications of rAd vectors have been made in an attempt to decrease immunogenicity and increase cell-specificity and efficiency of gene transfer. The first-generation vectors used in the rAd-OTC, rAd-p53, and similar studies were deleted only for portions of their E1a and (for some) E3 coding regions. These vectors contained all of the other viral genes. Although E1a functions as a master-switch for activation of other genes, these vectors were "leaky" for expression of other early and late genes, which may have increased their immunogenicity somewhat. Second generation vectors were designed that were deleted for E2a and E4 regions, and the latest helper-dependent (HD) Ad vectors are completely gutted, that is, possess no viral coding sequences (Yang et al., 1994; Engelhardt et al., 1994a,b; Gao et al., 1996; Raper et al., 1998; Alba et al., 2005; Schiedner et al., 1998). While these vectors have elicited much less adaptive cytotoxic T-cell responses, it remains to be seen whether there will be much effect on innate cytokine responses. More recently, there has been considerable effort made to modify the Ad capsid, either by utilizing other Ad serotypes or by making targeted mutations with insertion of specific ligands to target particular cell-surface receptors (Wickham et al., 1997; Krasnykh et al., 1998; Dmitriev et al., 1998; Romanczuk et al., 1999). Increasing the cell-specificity of Ad attachment could have a major effect on innate immunity

and increase the efficiency of gene transfer, thus decreasing the dose that would be needed for the desired effect. Once again, the clinical safety and efficacy of such modified vectors remains unproven.

Much as with rAd vectors, rAAV vectors have been subjected to targeted mutation. Receptor-specific ligands have been inserted into specific regions of the capsid gene so that they are then displayed on the surface of the capsid (Wu et al., 2000; Nicklin and Baker, 2002; Loiler et al., 2003, 2005; Nicklin et al., 2003; White et al., 2004; Work et al., 2004). Work is also progressing with rAAV vectors that are based on newly available serotypes and genomic variants of AAV (Chao et al., 2000; Gao et al., 2002; Rabinowitz et al., 2002). The first 10 years of clinical rAAV gene therapy relied on serotype 2 vectors. These vectors benefited from the natural biology of AAV, a non-pathogenic human parvovirus that can infect non-dividing cells and remain latent for prolonged periods, predominantly in an episomal state (Afonse et al., 1996; Flotte and Berns, 2005). AAV appears to persist in cells without causing insertional mutagenesis and does not trigger a robust innate response. It has a very limited packaging capacity, however (approximately 5 kb), and the original AAV2 is relatively inefficient at infecting a number of key cell types. Studies are now underway in the US and Europe with rAAV1 vectors, which have been shown to be nearly 1,000-fold more potent for murine muscle transduction than rAAV2 vector (Chao et al., 2000; Lu et al., 2006). Over 100 capsid variants are now known to exist (Gao et al., 2002, 2003). These vary widely in tissue tropism and may have differences in their ability to trigger innate and adaptive immune responses.

Finally, rAAV has been subjected to in vitro evolution, using DNA shuffling between available serotype sequences to create a library of novel genomic variants (Schaffer and Maheshri, 2004; Koerber et al., 2006; Maheshri et al., 2006; Perabo et al., 2006). With the advent of robust methods to examine the crystal structure of such mutants, further in vitro evolution and serotype selection has been targeted to sites that are known to be displayed on the surface and with known effects on vector stability or infectivity (Fig. 1; Wu et al., 2006). Serial passage of such mutant libraries on a target cell type of interest, or even a target tissue in a mouse model, has allowed for selection of mutants with increased specificity and efficiency and decreased susceptibility to neutralizing antibodies.

Work is also progressing with clinical applications of lentivirus, herpesvirus, and newer non-viral gene therapy vectors. Lentivirus vectors offer similar advantages to the

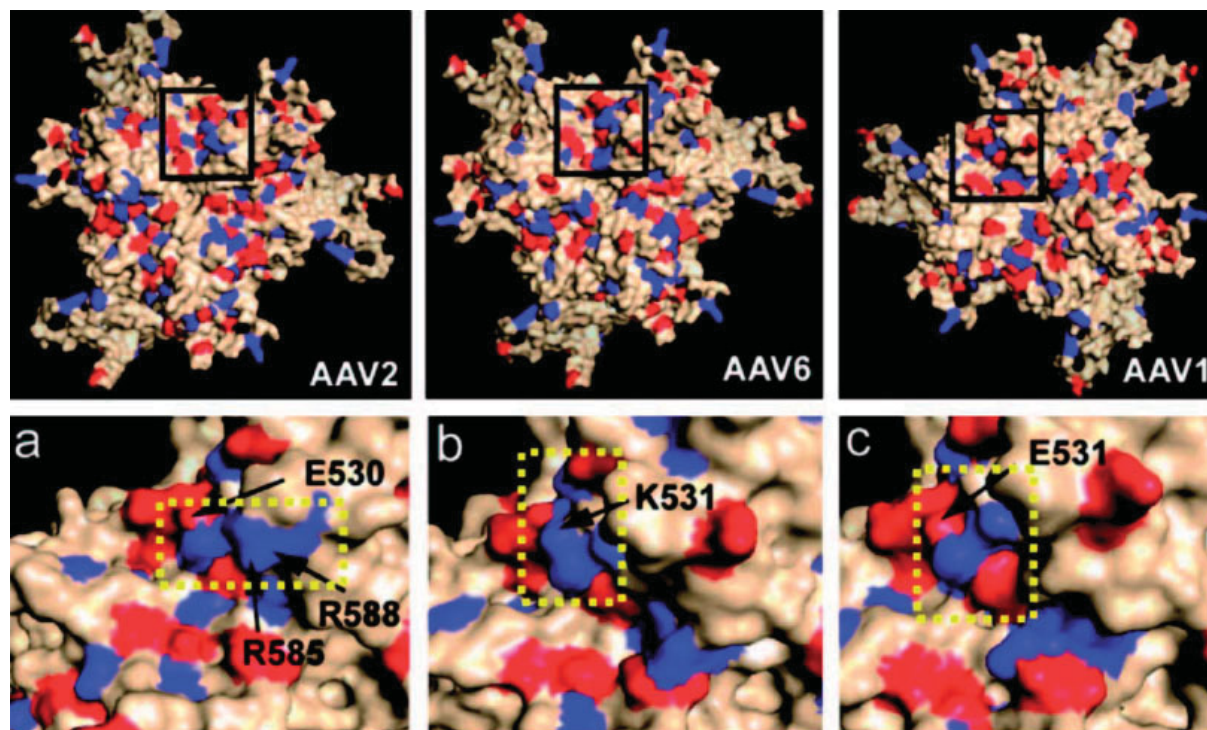


Fig. 1. Mapping of critical capsid mutant residues to the AAV2-VP3 crystal structure. (Reproduced with permission from Wu, et al., *J. Virology* 2006; 80:11393–11397.) The three-dimensional surface maps and charge distribution of a VP3 trimer (one face of the icosahedral capsid) are compared between AAV2, AAV6, and AAV1 (parts a, b, and c, respectively).

gamma-retrovirus vectors, in that they mediate long-term integration of the therapeutic transgene, but unlike gamma-retroviruses, they do not require cellular mitosis in order to gain access to the host genome for integration. They also are thought to share the potential for insertional mutagenesis with subsequent carcinogenesis, although this has not been observed in the limited number of clinical trials done to this point. They have also been successfully targeted by pseudotyping alternative surface glycoproteins for display on the lipid envelope of the vector.

Herpes simplex virus (HSV)-based vectors are large DNA-virus vectors that may trigger innate and adaptive immunity like rAd vectors, but they have a longer-term expression profile and can also infect non-dividing cells. These vectors have also gone through a series of iterations, including HSV amplicon vectors, which are HD and HSV recombinant vectors, which are conditionally replication deficient, but still may express low levels of the HSV proteins.

The non-viral systems that have been used for gene transfer are numerous (Niidome and Huang, 2002). Naked DNA alone is capable of significant gene transfer to skeletal muscle when directly injected (Wolff et al., 1992), and in mice can also transduce the liver when injected rapidly under high pressure into the subdiaphragmatic venous system (Knapp and Liu, 2004; Zhang et al., 2004). Naked plasmid DNA has been used for recombinant vaccine studies and for direct injection of a variety of genes into tumors. Numerous cationic liposomes have been used in clinical gene transfer, as well, either alone or as mixtures with neutral “co-lipids.” DNA conjugates with other poly-cationic molecules, such as polyamines, poly-lysine have also been used in clinical studies, either as a compacted DNA

alone or in a complex with targeting peptides (Konstan et al., 2004). The latter modifications are in many ways analogous to the capsid targeting strategy described above for viral vectors. In addition, the safety of non-viral vector have been improved by the remove of CpG motifs which were the primary cause of innate responses through their interaction with toll-like receptors on the cell surface (Tan et al., 1999; Liu et al., 2004).

Current and Future Disease Targets for Gene Therapy

The disease states that have been approached with gene therapy are now widely diverse, including autosomal recessive single gene disorders (CF, ADA-SCID, hemophilia), autosomal dominant disorders, many forms of cancer, HIV and other infectious diseases, inflammatory conditions, and intractable pain (Table I). In most instances, the nature of the disease may direct the vector to be used, since it may dictate a specific desired cell type, expression level, and duration of expression that will be required for therapeutic effect. In general, genetic diseases will require long-lasting gene expression, and so may indicate the use of a gamma-retrovirus, lentivirus or rAAV vector; while a tumor vaccine or cytotoxic gene approach to cancer may require a short burst of gene expression, such as might be produced by a rAd or non-viral vector.

For a disease to be a good candidate for gene therapy, the role of the therapeutic gene in disease pathophysiology must be clearly understood. In this context, single gene disorders have a clear advantage, although assumptions about the desired cell type to be targeted and the appropriate timing of gene transfer have not always proven to be accurate. Single gene

disorders may also present some disadvantages, particularly in patients with null mutations who may produce immune responses to the transgene product. Conversely, there is not likely to be an immune response from transient overproduction of a natural angiogenic factor in ischemic vascular disease, for instance, but it may not always be reasonable to expect a multifactorial disease to respond to overexpression of a single gene. Likewise, overexpression of a tumor-suppressor gene may have some effect in cancer, but could still be short of curative.

It seems likely that in the future more gene therapy targets will be identified, primarily as a result of the rapid ability to identify specific gene associations with human diseases, particularly after the completion of the Human Genome Project. This includes both single gene diseases, and multifactorial diseases in which complex networks of gene and environmental effects are elucidated, and key genetic determinants of disease are identified within those pathways. The future remains uncertain regarding the ultimate clinical impact that gene therapy will have in each of those very distinct situations.

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