

Gene therapy for immune disorders: Good news tempered by bad news

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After a dozen years of human gene therapy trials characterized by minimal gene correction and disappointing clinical impact, the field of gene therapy received some good news in 2000. Infants with X-linked severe combined immunodeficiency who received retroviral gene addition to cells from their bone marrow developed impressive immune reconstitution. During the following 2 years, additional patients were treated and the news was even better—babies receiving gene therapy had sustained T-cell production and in several cases developed better cell function than most patients treated with standard bone marrow transplants. Unfortunately, bad news followed. Three of the patients experienced leukemic T-cell expansions, found to be associated with retroviral insertions into genomic DNA. Where does the field stand today? (*J Allergy Clin Immunol* 2006;117:865-9.)

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Gene therapy for 2 types of severe combined immunodeficiency (SCID) has been highly successful, but a substantial risk of leukemia in gene-targeted cells has emerged. Where does the field stand now, and what are the challenges it faces?

Let us start with some definitions. First, what is gene therapy? Several types of genetic modifications have been proposed and are under study with the aim of treating a variety of diseases. However, the most technically feasible and only approach used to date in human primary immunodeficiencies is the addition of a correct copy of a gene to the somatic cells of an individual with an inherited genetic deficiency. Note that this sort of gene therapy is not going to have any effect on the germ cells, and thus any modifications will not be passed on to offspring.

Strategies for transfer of genetic material into target cells need to be based on a full understanding of the biologic characteristics of each particular disease under

Abbreviations used

ADA: Adenosine deaminase
BMT: Bone marrow transplantation
 γ c: Common γ chain of cytokine receptors
HSC: Hematopoietic stem cell
LMO-2: LIM domain only 2
LTR: Long terminal repeat
SCID: Severe combined immunodeficiency

study. For example, gene therapy for cystic fibrosis might be focused on delivering correct copies of the *CFTR* gene to cells lining the small bronchioles in the lung to provide expression of the cystic fibrosis transmembrane conductance regulator protein, restoration of ion channels, and normalization of secretions. Diseases of hematopoietic cells need to take into account the constant turnover of blood cells, which are produced throughout life by differentiation from a small pool of self-renewing hematopoietic stem cells (HSCs) in the bone marrow that give rise to all blood lineages. Correction of diseases of blood leukocytes can be achieved by means of bone marrow transplantation (BMT) from a healthy, HLA-matched allogeneic donor, and therefore cures can likewise be sought through transfer of a correct copy of the defective gene into a population of autologous HSCs. For a correct gene copy to be passed on to successive generations' daughter cells, it must be integrated into the human genome. Retroviruses have been developed as vectors for corrective genes because they characteristically insert the genes they carry into the chromosomal DNA of target cells and are thus replicated along with chromosomal DNA when cells undergo mitosis.

The term *gene therapy* implies a beneficial effect in human subjects; until recently, the term *gene transfer* was preferred as a less optimistic term in a field that experienced much hype with generally disappointing clinical results through the 1990s. However, now infants with SCID have been successfully treated by transferring correct gene copies into hematopoietic cells from bone marrow by means of retroviral vectors.

Let us consider why SCID has been a pilot disease for treatment with currently available gene-transfer methods. SCID can be caused by defects in many different genes, but all genotypes of SCID share a profound lack of both T-cell and B-cell immune function.^{1,2} Infants affected with these genetic disorders experience recurrent and opportunistic infections beginning at 2 to 4 months of age, when levels of maternally derived IgG wane. Without treatment, SCID is generally fatal in the first year of

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life.^{1,2} BMT from an HLA-matched sibling is the treatment of choice for infants with SCID.³⁻⁵ Although effective methods using T cell-depleted HLA-haploidentical marrow from a parent or HLA-matched unrelated donor marrow have been developed for children with SCID who do not have a matched sibling,⁶⁻⁸ the survival rate is not as high, and some of the patients who initially have T-cell function experience waning immune reconstitution, leading to growth retardation and other evidence of chronic illness.⁹

The fact that BMT can effect a permanent cure for SCID indicates that reconstitution of functional HSCs by means of gene transfer to autologous cells should also be possible. Because HLA-matched related donors are not available for most patients with SCID, gene transfer to autologous cells is a desirable therapeutic option. Bone marrow can be harvested, cultured *in vitro* with retroviruses encoding the therapeutic gene product, and returned to the patient by means of intravenous infusion, a technology that has been developed over the past decade.

The genes for several types of SCID are known and well studied, in particular the X-linked *IL2RG* gene, encoding the common γ chain of cytokine receptors (γ_c), and *ADA*, encoding the enzyme adenosine deaminase (ADA). These genes account for about 50% and 15% of all SCID cases, respectively.^{1,2} Prior experiments indicated that both genes are expressed in all blood cells, and neither is tightly regulated, suggesting that expression of either γ_c or ADA driven by the unregulated, non-tissue-specific promoter sequences contained within the retrovirus 5' long terminal repeat (LTR) region would not be harmful. A major hurdle for gene therapy in general is immune reactions against the corrected gene product or the cells that make it, but this complication is not expected in patients with SCID, given their severely compromised immunologic function. Finally, in both of these SCID genotypes, an *in vivo* selective advantage for survival and proliferation has been demonstrated by cells bearing correct, as opposed to mutant, copies of the disease gene.² Thus even with the relatively inefficient gene-transfer methods available today and even though true HSCs are rare in the bone marrow and impossible to completely purify, therapeutic effects in treatment trials were anticipated.

Indeed, since the recent series of trials starting in 1999, successful treatment of SCID with retroviral gene therapy has been reported in more than 20 infants with *IL2RG* or *ADA* mutations.¹⁰⁻¹³ In *ADA* deficiency, both withholding administration of exogenous *ADA* enzyme and treatment with busulfan before infusion of corrected cells were used to give an extra advantage to the gene-corrected cells. However, patients with X-linked SCID have not required chemotherapy, allowing this treatment to be used even in patients who are gravely ill with infections and judged unlikely to survive marrow-suppressive treatment. Graft-versus-host disease predictably did not occur, and immune reconstitution has been achieved in the patients who received greater than 3 million corrected CD34⁺ cells per kilogram of body weight. Many of the treated children

not only have functional T cells but also have made antibodies after childhood vaccines and natural infections. Their outcomes compare favorably with those of recipients of non-HLA-identical allogeneic BMT, after which B-cell correction has been problematic.⁹

Thus although it remains to be proved whether lifelong cures have been achieved, gene therapy has finally become a beneficial treatment for at least some patients with SCID caused by γ_c or *ADA* deficiency who have no matched sibling donor.

In late 2002, 2 children with X-linked SCID, who had received gene therapy as infants almost 3 years previously and who had achieved immune reconstitution, were diagnosed with lymphoproliferative disorders similar to lymphocytic leukemia. The common link in both patients was that their leukemic T-cell clones contained the gene therapy vector inserted either into the promoter or first intron of the same proto-oncogene, *LMO2*.^{14,15} Furthermore, in both cases the LIM domain only 2 (*LMO-2*) protein appeared to be produced abnormally in the leukemic clones, presumably resulting from these vector insertional events. *LMO-2* had already been linked to murine and human lymphocytic leukemias,¹⁶ suggesting that gene vector insertional mutagenesis activated *LMO-2* and contributed to the development of leukemia in these patients with SCID. Both patients received chemotherapy, resulting in a prolonged remission in one patient with retention of the immune correction, but the other child died in late 2004 despite a matched unrelated donor BMT. In January 2005, a third child was reported to have a lymphocytic leukemia (online report from the French regulatory agency, <http://agmed.sante.gouv.fr/hm/10/filcoprs/050103en.htm>), also about 3 years after successful immunorestorative gene therapy. In this case a vector insertion near the *LMO2* gene was found also, but its significance to the lymphoproliferative disorder was less clear than in the previous cases because the leukemic cells of this patient also had 3 additional vector insertions near proto-oncogenes.¹⁷

In all 3 cases of leukemia in the French trial, the malignant clones also had cytogenetic abnormalities, suggesting that events in addition to vector insertion into proto-oncogenes were required for progression to leukemia. No cases of leukemia have been reported in recipients of gene therapy for *ADA*-deficient SCID.

What is our current understanding of leukemia in HSC gene therapy for single-gene disorders? All of the clinical trials to date have used γ retroviruses, which are simple retroviruses (with few or no modifier elements) exemplified by Moloney murine leukemia virus. Another major class of retroviruses is the lentiviruses (complex viruses containing many regulatory elements), which are exemplified by HIV.¹⁸ Even the earliest discussions of gene therapy in the 1980s recognized the potential of insertional mutagenesis to cause cancer either by activating a gene that promotes cell growth or by inactivating a protective gene (eg, genes that induce apoptosis or provide checks on progression of the cell cycle). Wild-type versions of Moloney murine leukemia virus and Harvey murine

sarcoma virus were known to cause hematologic or solid tumors in experimental animals. Nonetheless, it was believed that versions of these viruses from which the envelope and much of the *gag-pol* sequence were removed would be safe for gene therapy because they were incapable of self-replication and thus could not set up a chain reaction of continuing insertions.¹⁸ Indeed, in many animal studies and the first decade of human clinical trials of gene therapy using replication-incompetent vectors, no cancer was observed. Furthermore, in 1992, a lymphoma in a primate that received HSC gene transfer with a batch of vector contaminated with replication-competent helper virus reinforced the idea that replication-competent virus might be necessary to cause cancer.¹⁹ In fact, it was only within the past year that a myeloid sarcoma was observed in a monkey treated with *ex vivo* HSC gene therapy where the vector was clearly free of replication-competent helper virus.²⁰ Even in this case it appears that extreme oligoclonality of the gene-marked cells plus the animal's subsequent treatment with busulfan chemotherapy might have been required for the progression to transformation.

The first unequivocal demonstration that replication-incompetent γ retrovirus could cause leukemia in early 2002 described a transplantable myeloid leukemia in a mouse model of HSC gene transfer.²¹ The leukemic clone contained a single vector copy integrated into the murine ecotropic viral integration site 1 (*Evi1*) gene active in early myeloid development; the integration event turned on production of the ecotropic viral integration site 1 gene product. The vector in this study encoded a version of the human nerve growth factor receptor with the extracellular and transmembrane domains present but with the intracellular signaling domain deleted. The authors proposed a model in which excessive expression of a cell growth–signaling element (the truncated nerve growth factor receptor complexed with other cell-signaling molecules) achieved synergy of cell proliferation in a clone in which insertional mutagenesis had also activated ecotropic viral integration site 1. This excessive proliferation predisposed the clone to additional events, such as chromosomal rearrangements, that led to frank leukemia. Notably, a long-term follow-up study of nonhuman primates with high-level *ex vivo* gene marking of HSC showed a high frequency of vector inserts in the *EVII* locus without progression to leukemia, supporting the notion that insertions at this locus might provide a growth advantage to such clones but that additional factors are likely to be necessary for progression to leukemia.²² Taken together, such data present a mixed message: γ retrovirus–mediated gene therapy, in which the therapeutic gene is not itself a growth-promoting factor, might be more risky than we used to think but perhaps not quite as dangerous as recent events might be leading us to fear. However, new information from many investigators continues to accumulate, so that even this statement might seem out of date in the near future.

A mouse retrovirus-tagged cancer gene database (<http://RTCGD.ncicrf.gov>) lists insertion sites in tumors arising spontaneously in mice chronically infected with

replication-competent retroviruses.²³ There is a predominance of insertions associated with known proto-oncogenes relative to statistically predicted frequencies if insertion into the total genome is random, and some correlations between tumor types and insertion sites are emerging. Certain associations of retroviral insertion sites in the same tumor cell suggest that inserts in more than one proto-oncogene or inserts in a proto-oncogene together with inserts in genes encoding growth-promoting signaling molecules might be oncogenic. Of note were several leukemias with retroviral insertions at *lmo2*, 2 of which also had insertions in the same cell at *il2rg*, as well as third leukemia with an insertion in *il2rg* together with an insertion at another proto-oncogene. These mouse data again support the notion that the leukemias arising in the 3 patients with X-linked SCID might have derived from a growth-promoting synergy between the therapeutic gene, *IL2RG*, encoded in the vector and the insertional activation of *LMO2* or another proto-oncogene.

The development of leukemia is a setback for gene therapy as a treatment for X-linked SCID and a concern for use of retroviruses in other types of HSC-directed gene transfer, including ADA SCID. However, the published outcome data from standard BMTs for SCID indicate that we still have to strive for improvement in our treatment of these patients. Overall mortality of infants with SCID remains significant in patients who present with life-threatening infections that complicate BMT. Even after successful BMT, fewer than half of the patients with X-linked SCID have adequate B-cell function, and thus the majority remain dependent on immunoglobulin replacement. Moreover, some patients experience decreases in production of new T cells several years after reconstitution by BMT⁷; the hypothesis that gene-corrected autologous HSCs might provide more durable immunoreconstitution will require long-term follow-up to be tested.

One approach to improving outcomes for patients with SCID is to detect it earlier, so that treatment can be instituted before serious infections occur. Survival of patients who lack a related HLA-matched donor and receive BMT at greater than 3.5 months of age is around 70%, whereas those given diagnoses prenatally or at birth because of a positive family history can have survival rates approaching 95% when treated at 3.5 months or earlier.^{8,24} Universal screening of newborns for SCID could thus potentially save lives, and methods for screening are under investigation.²⁵

Given our current information, what might be done to make gene therapy safer? After the reports of leukemia in patients receiving X-linked SCID gene therapy in France, there were public forums in the United States sponsored by the US Food and Drug Administration and by the National Institutes of Health Office of Biotechnology Activities in the context of meetings of the Recombinant DNA Advisory Committee. Clinical and basic research data about gene transfer and insertional mutagenesis were discussed. Both regulatory bodies came to similar conclusions. They agreed that insertional mutagenesis played

TABLE I. Status of immunodeficiency gene therapy**Present**

Carefully weigh risks and potential benefits for each patient.
 Chose patients most at risk from their disease process and least at risk from adverse effects of gene therapy.
 Recognize that therapeutic genes with growth-promoting properties might contribute to oncogenesis.
 Monitor insertion site profile, checking for clonal expansion of transduced cells.

Future possibilities

Develop vectors that reduce targeting of transcriptional start sites (lentivectors) or active genes.
 Use self-inactivating vectors, incorporate insulators, and include suicide genes.
 Improve methods to purify and expand undifferentiated HSCs *in vitro* so that vector insertion sites can be monitored before reinfusion.
 Develop safe and efficient methods for correction of genomic mutations rather than gene addition.

an important role in the development of the leukemias in the patients with X-linked SCID and that additional risks particular to X-linked SCID might arise from the circumstance that the therapeutic gene *IL2RG* encodes a growth factor. For the present (Table I, top), the US Food and Drug Administration ruled that HSC gene therapy clinical trials may proceed but that there must be informed consent concerning the severe adverse events of leukemias. In treatment of other gene deficiencies, like ADA-deficient SCID, in which the therapeutic gene is not itself a growth factor, gene therapy might be less risky, whereas for X-linked SCID, one must be mindful of potentially greater risk. Gene therapy for X-linked SCID has not been completely prohibited but is now restricted to patients who are unlikely to survive allogeneic BMT or have already undergone standard BMT treatment but do not have satisfactory T-cell immunity. In ADA-deficient SCID the relative risks and benefits of polyethylene glycol (PEG)-ADA, allogeneic BMT, and retroviral gene therapy need to be considered on an individual basis. Gene therapy has not been shown to cause leukemia in ADA SCID to date, but too few patients have been treated to rule out this risk. Moreover, the successful gene therapy protocol requires stopping or withholding protective PEG-ADA along with cytoablative conditioning.¹³ In addition, investigators conducting gene therapy trials must monitor the patients' vector insertion sites to look for emergence of any clone carrying a particular insertion.

The occurrence of leukemia in recipients of retroviral gene therapy in SCID translational clinical trials has identified basic research questions that need to be fully addressed to make gene therapy safer in the future, as summarized in the lower portion of Table I. Safer approaches might grow out of recent investigations. For example, it is now clear that some classes of vector have intrinsic patterns of insert site selection in the genome that are "safer" than others. Several investigative groups have shown that γ retroviruses not only have preference

for inserting near genes (particularly genes that are active in the cells) relative to intergenic regions of DNA but that there is even a particular preference for the promoter and the 5' regions of a gene.^{26,27} By contrast, HIV and other lentiviruses, which also prefer to insert themselves near active genes, do not have this preference for the "front end" of genes. Thus one maneuver to make vectors safer might be the use of replication-incompetent vectors engineered from lentiviruses. These studies also suggest that increased knowledge about why these insertional preferences occur might allow engineering of vectors that insert in intrinsically safer parts of the genome.

Another maneuver to improve the safety of vectors might be to cripple the virus LTRs that are a likely source of activation of the promoter regions of nearby host cell genes. Because vector integration into a target cell genome involves a complex process by which the 3' LTR sequence ends up replacing the 5' LTR sequence in the integrated vector,¹⁸ a number of investigators have developed lentivirus constructs with crippled 3' LTRs that can be packaged and will infect target cells but after integration have no 5' LTR activity. Such self-inactivating vectors must have an internal promoter, but another positive aspect of this approach is that the internal promoter can be tissue specific.²⁸ Of course this internal promoter, even if it is not a virus promoter, is likely to have enhancer elements that can in theory activate nearby genomic promoters after vector insertion. There are naturally occurring insulator elements, sequences that limit cross-talk between genomic elements, that can not only protect the therapeutic gene in a vector from being silenced but might also prevent the enhancer elements of the vector from activating nearby genes.^{29,30}

In addition to vector improvement, better understanding of stem cell differentiation might lead to the ability to manipulate totipotent HSCs *ex vivo* so that they can be transduced, expanded, and tested for dangerous insertions before infusion into patients. Similarly, improved abilities to culture purified HSCs could make possible direct methods of correcting genomic mutations, which are presently too inefficient and nonspecific for clinical use. In theory the safest system of gene therapy should simply repair the mutation causing a monogenic inherited disease *in situ*. Until recently, this type of site-directed recombination repair could only be accomplished at extraordinarily low efficiency, but new methods of site-directed recombination can introduce a specific nucleotide change at a specific site in 1% to 5% of target cells.³¹ The method relies on plasmids that encode engineered recombinant protein domains with a series of DNA-binding zinc finger domains to bind a unique DNA sequence in the cellular genome. Additional protein domains in the construct encode an endonuclease that cuts the genomic DNA at that site, thereby triggering intrinsic cellular DNA repair processes. By also introducing into the target cell an excess of DNA template matching the site of interest but having a wild-type sequence, a high likelihood correction upon repair of the DNA break is achieved. This very

preliminary work in cultured cell lines has not been evaluated in animals yet.

Like many new therapies in medicine, gene therapy is a new modality of treatment with both pros and cons. It has become a powerful and successful clinical tool, while at the same time presenting adverse effects that must be addressed before it can come into wider use.

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