doi:10.1016/S1525-0016(03)00212-0

American Society of Gene Therapy (ASGT) Ad Hoc Subcommittee on Retroviral-Mediated Gene Transfer to Hematopoietic Stem Cells

Donald B. Kohn, Michel Sadelain, Cynthia Dunbar, David Bodine, Hans-Peter Kiem, Fabio Candotti, John Tisdale, Isabelle Riviére, C. Anthony Blau, Robert E. Richard, Brian Sorrentino, Jan Nolta, Harry Malech, Malcolm Brenner, Kenneth Cornetta, Joy Cavagnaro, Katherine High, and Joseph Glorioso

Gene transfer using retroviral vectors has been under clinical study for more than 12 years [1]. Many studies have targeted hematopoietic stem cells (HSCs) as a potentially enduring and renewable source of gene-modified blood cells for the treatment of specific genetic diseases, cancer, leukemia, and HIV-1 infection [2]. Although initial studies were hampered by very low levels of gene transfer to HSCs, incremental progress has been realized in the efficiency of gene transfer to HSCs. These advances have culminated in the report of clinically significant restoration of immunity in patients with the X-linked form of severe combined immune deficiency (XSCID) by Alain Fischer, Marina Cavazzana-Calvo, and colleagues at the Hôpital Necker Enfants Malades in Paris [3]. Their study and those conducted by Adrian Thrasher and colleagues at the Great Ormond Street Childrens Hospital in London for XSCID and by Claudio Bordignon and colleagues at the Hospital San Raffaele in Milan for children with SCID due to deficiency of adenosine deaminase (ADA) provide incontrovertible proof that gene therapy can ameliorate genetic diseases [4,5].

Tragically, two subjects of the French trial for XSCID developed T-cell leukemia more than 2 years after the gene transfer procedure [6]. In both cases the leukemia cells contained a single copy of the intact retroviral vector inserted in or near the locus of the *LMO2* gene. The prompt and responsible reporting of these serious adverse events to the gene therapy community worldwide led to the placement of clinical holds on similar studies while the causes are investigated and the risks and benefits of this approach are reconsidered.

The board of directors of the American Society of Gene Therapy (ASGT) discussed ways in which members of ASGT could assist in the response to these adverse events to help explain the causative mechanisms and to define means of minimizing risks in future studies while maintaining the clinical benefits. It was proposed that members of the ASGT could help to educate fellow scientists, as well as regulatory agencies, advisory boards, and the general public, by gathering the relevant data on preclinical and clinical studies using retroviral vectors to transfer genes to HSCs. Although some of this information has been published in the scientific literature, it had not before been compiled in a comprehensive manner. Beyond initial publications regarding a cohort of animals or patients, longer term follow-up information on toxicity, persistence of genetically modified cells, or efficacy has not generally been available in the literature. Contrasting the specific features of the XSCID study against the background of other studies that have not included this complication may shed light on contributory elements.

At the direction of the board, an ad hoc subcommittee was established to carry out these activities (Table 1). During January and February 2003, the subcommittee members and many other contributors from the gene therapy research community provided, gathered, and compiled the available information. Three specific areas were examined: (1) the extensive studies in murine models of gene transfer into HSCs, including normal mice and gene knockout models of genetic diseases, as well as studies in transgenic mice, immune-deficient mice, and mice infected by wild-type, replication-competent retroviruses (RCRs); (2) studies in large animal models, such as nonhuman primates and dogs, that mirror more closely the relevant human physiology and HSC biology than do rodents; (3) the clinical trials that have been conducted. We here present a condensed version of the findings, which have been publicly posted at the ASGT website (www.asgt.org). Readers should refer to the website for more details and full references.

Although the potential risk of retroviral vectors causing cellular transformation through insertional oncogenesis was recognized, it was not reported in preclinical studies other than those complicated by the presence of RCRs, and was generally thought to be a rare and primarily theoretical complication. Wild-type RCRs are known to cause insertional oncogenesis mainly when infecting newborn mice, wherein the relative immune incompetence of the hosts allows high-level viral replication leading to massive numbers of integration events and thus eventually an individual insertion adjacent to a cellular proto-oncogene, resulting in inappropriate activation. In the absence of RCRs, the clinical preparations of retroviral vectors that were tested for the absence of replication were thought to be at relatively low

TABLE 1: American Society of Gene Therapy (ASGT) ad hoc subcommittee on retroviral-mediated gene transfer to hematopoietic stem cells and conscripted contributors

Area	Subcommittee members		Contributors	
Murine			Tony Blau	Univ. of Washington
	Michel Sadelain	MSKCC		
	David Bodine	NHGRI	Jan Nolta	Washington Univ.
	Fabio Candotti	NHGRI	Isabelle Riviére	MSKCC
			Robert E. Richard	Univ. of Washington
			Brian Sorrentino	SJCRH
			John Tisdale	NIDDK
			Makoto Otsu	U. Tsukuba, Japan
Large animals			Claire Soudais	Hôpital Necker, France
	Cynthia E. Dunbar	NHLBI	Robert Donahue	NHLBI
	Hans-Peter Kiem	FHCRC	John Tisdale	NIDDK
	David Bodine	NHGRI	Stephanie Sellers	NHLBI
			Harry Malech	NIAID
			Jennifer Puck	NHGRI
			Robert Andrews	FHCRC
			Bobbie Thommason	FHCRC
			R. Paul Johnson	NERPRC/Harvard
			Chris Porada	Univ. of Nevada
			Esmail Zanjani	Univ. of Nevada
			Yutaka Hanzono	Jichi Medical School, Japan
			Keiya Ozawa	Jichi Medical School, Japan
Clinical trials				
	Donald Kohn	USC	James Croop	Indiana Univ.
	Kenneth Cornetta	Indiana Univ.	Stan Gerson	Case Western Reserve
	Malcolm Brenner	Baylor	Ronald Mitsuyasu	UCLA
	Harry Malech	NIAID	Cynthia Dunbar	NHLBI
			John Barranger	Univ. of Pittsburgh
			Johnson Liu	NHLBI
			Chris Walsh	Univ. of N. Carolina
			Edwin Horowitz	SJCRH
			Maria Cavazzanna-Calvo	Hôpital Necker, France
Other				
	Joy Cavagnaro	Access BIO		
	Katherine High	CHOP/Univ. of Pennsylvania		
	Joseph Glorioso	Univ. of Pittsburgh		

MSKCC, Memorial Sloan Kettering Cancer Center; NHGRI, National Human Genome Research Institute; SJCRH, St. Jude Children's Research Hospital; NIDDK, National Institute of Diabetes & Digestive & Kidney Diseases; NHLBI, National Heart, Lung, and Blood Institute; FHCRC, Fred Hutchinson Cancer Research Center; NIAID, National Institute of Allergy and Infectious Diseases; NERPRC, New England Regional Primate Research Center; USC, University of Southern California; UCLA, University of California, Los Angeles; CHOP, Children's Hospital of Philadelphia.

risk for causing insertional oncogenesis, because most cells seemed to contain no more than one proviral genome. Nevertheless, this potential risk of insertional mutagenesis has been a standard element contained in the informed-consent documents provided to subjects of clinical trials using retroviral vectors.

doi:10.1016/S1525-0016(03)00212-0

TABLE 2: Serious adverse events reported in long-term murine chimeras engrafted with retrovirally transduced hematopoietic cells

Transgene	Transcriptional control element	Serious adverse event	Transformation	Reference
IL2	MoMLV LTR	Intense hepatic lymphocytic infiltration	−	Kuhr, Exp. Hematol, 2000
IL3	MPSV LTR	Fatal non-neoplastic myeloproliferation	−	Chang, Blood, 1989
IL6	MoMLV LTR	Extensive plasma cell infiltration	−	Brandt, J. Clin. Invest., 1990
G-CSF[a]	MPSV LTR	Extensive neutrophilic infiltration	−	Chang, J. Clin Invest, 1989
MDGF[a]	MSCV LTR	Pancytopenia, myelofibrosis, osteosclerosis	−	Yan, Blood, 1995
ADA	MoMLV LTR	RCR (no linkage to ADA)	−	Belmont, Mol. Cell Biol., 1988
c-mpl	MSCV LTR	Anemia, thrombocytopenia	−	Yan, Exp. Hematol., 1999
TGF βR2	MSCV LTR	Marked myeloid expansion	−	Shah, J. Immunol., 2002
Δ4 Notch ligand	MSCV LTR	Fatal lymphoproliferative disease	−	Dorsch, Blood, 2002
TPO	MPSV LTR	Pancytopenia, undifferentiated leukemia	+	Villeval, Blood, 1997
MDR1[a]	HaMSV LTR	Myeloproliferative syndrome, acute leukemia	+	Bunting, 1998; Bunting, 2000
FLT3 ligand	MSCV LTR	B-cell or myeloid tumors	+	Hawley, Blood, 1998
CD40[a] ligand	MoMLV LTR	Lymphoblastic thymomas and lymphomas	+	Brown, Nat. Med., 1998
BCL2	MPSV LTR	Pre-B lymphomas	+	Davidson, Exp. Hematol., 1999
ΔLNGFR	MoMLV LTR	Myeloid leukemia	+	Li, Science, 2002

[a]Current approved gene names are as follows: *CSF3* (*G-CSF*); *PPBP* (*MDGF*); *ABCB1* (*MDR1*); *TNFRSF5* (*CD40*).

MURINE STUDIES

The report on existing studies in murine models was coordinated by Michel Sadelain and David Bodine, with separate sections written by invited experts.

John Tisdale (NIDDK), Isabelle Riviére (MSKCC), and Michel Sadelain (MSKCC) reviewed the frequency of tumor formation associated with retroviral-mediated gene therapy in murine studies. After providing a broad survey of the literature, they reviewed 157 publications. Short-term studies (<50 days) and studies investigating known oncogenes were excluded. The combined number of mice is 5436, of which 4846 were followed at least 3 months and 1496 at least 6 months. Based on a minimum of 3 months of follow-up, the average publication counts 30 mice, of which 30% are followed 6 months or more, and 30% are secondary transplant recipients.

Regrettably, most studies do not include specific studies or statements on the health status of long-term chimeras. Serious adverse events (SAEs) consisting of hematopoietic anomalies were reported in 15 studies, including 6 with malignant transformations (Table 2). All but 2 of these SAEs are clearly linked to the specific function of the vector-encoded transgene. The two exceptions are a report of an RCR, first detected in serum and eventually traced back to the packaging cell line used in this study [7], and a report of myeloid leukemia in mice engrafted with hematopoietic cells expressing a truncated form of the human low-affinity growth factor receptor (ΔLNGFR). The latter is the sole documented case of insertional oncogenes in the literature [8]. Although the authors of this study proposed that ΔLNGFR may have contributed to the transformation [8], this possibility remains unproven to date [9].

It is of great interest, but much uncertainty, to infer a frequency of insertional oncogenesis in mice based on these numbers. Many critical variables would have to be taken into consideration, in addition to the vector characteristics: the number of engrafted hematopoietic cells, the transduction efficiency (and hence the number of integration events per recipient), the cytokine treatment applied to the graft, the conditioning regimen administered to the recipient, the donor's and host's age and genetic background. On the basis of the single positively identified case of insertional oncogenesis, the risk of tumor formation in mice followed for at least 3 months can be estimated to be 1 in 4846. However, this frequency is likely underestimated because of possible underreporting and insufficient follow-up. The frequency based on a follow-up of at least 6 months is 1 in 1496. In only a handful of reports is the duration of survey extended to 80–120 weeks on the basis of investigations in long-term secondary or tertiary transplants [10–12]. Ultimately, the determination of the risk of insertional oncogenesis will require well-designed prospective studies.

Studies involving transduction with drug resistance genes, genes allowing selective proliferation and amplification of cells, or the homeobox gene *HoxB4,* were re-

TABLE 3: Recommendations for murine studies of gene transfer to optimize the yield of relevant information

- Hematological analyses and necropsies should be systematically carried out in long-term hematopoietic chimeras. It would be reasonable to request that preclinical studies used to support proposed gene therapy trials conduct a set of standard hematological analyses.
- A certain number of treated animals should be maintained for at least 1 year. The classic 4-month follow-up is minimally informative with respect to assessing oncogenicity.
- Long-term follow-up should be highly valued by reviewers and editors; papers should clearly indicate the number of mice and duration of follow-up (these parameters are often difficult to obtain).
- Therapeutic genes should be tested in relevant disease models.
- Preclinical animal studies should appropriately mimic the human trials. The experiments should attempt to use animals of an appropriate age, relevant gene transfer efficiencies, transduction conditions, cell doses, and conditioning regimens (eventually without myeloablation).
- Novel transgenes should be tested in transgenic mice as well as in hematopoietic chimeras. The effect of increased expression of the γc chain in the development of the T-cell leukemias is not known. More studies are needed to determine the need for tighter regulation of γc expression (temporal regulation, level differentiation stage restriction).
- Integration sites should be monitored, at least in the event of a myeloproliferative or lymphoproliferative disorder. Now that the mouse genome is publicly available, the determination of insertion location should become part of the experimental plan in selection studies. DNA should at least be stored from all animals upon sacrifice, so that later analyses can eventually be carried out in the setting of unexplained adverse events.
- The integration sites should be related to target cell type, transduction conditions, clonal expansion, duration of transgene expression, and toxicities.
- Investigators in the field of gene therapy should share and centralize the information with the goal of establishing a large database of integration sites to assess the frequency of retroviral integration sites and their risk.

viewed by Anthony Blau and Robert Richard (University of Washington). A wide variety of strategies have been used in an attempt to endow genetically modified stem cells with a selective advantage. The reviewers identified 28 papers representing data from 1464 individual mouse transplants. These animals were followed for an average of 23 weeks. The design of many of the presented studies involved the use of chemotherapeutic agents at doses for which toxicities were predictable. Bunting et al. described the only documented malignancy in mouse transplant studies employing in vivo selection. Mice transplanted with multidrug resistance (MDR)-transduced cells that had been expanded ex vivo before transplantation developed a myeloproliferative syndrome that could progress to acute leukemia [13]. The myeloproliferative syndrome was observed at a much lower frequency in the absence of prolonged ex vivo expansion [14]. Another report did not demonstrate the development of malignancy [15].

Studies in γC- and Jak3-deficient mice were reviewed by Fabio Candotti (NHGRI) and Brian Sorrentino (SJCRH). Five published studies were identified using the γc gene knockout model of XSCID, for a total of 55 mice. Additionally, at least 33 more mice have been studied in the Candotti laboratory. No tumors were reported in any of the studies investigating γc gene transfer. There are obvious differences between murine models and clinical trials. In retrospect, the murine models may not be as useful as previously thought in terms of predicting toxicity outcomes because of differences in transduction and transplantation protocols, the age of treated mice, as well as basic biological aspects of the gene defects. The longest reported follow-up is only ~11 months. A high incidence of T-cell leukemias has been observed in long-term Jak3$^{-/-}$ mice, without correlation with the retroviral transduction of the Jak3 cDNA (B.S., unpublished observations).

Jan Nolta (Washington University) reviewed tumorigenesis in transgenic mice and mice with congenital immune deficiencies. Even in the absence of any gene transfer, the incidences of leukemia and lymphoma are significantly higher in immune-deficient mice than in their normal counterparts. Thus, variables that predispose cells to chromosomal rearrangements, such as defects in DNA repair pathways and mutagens, have a much greater chance of causing neoplasia in immune-deficient mice. Leukemia and lymphoma also have increased frequencies in mice transgenic for interleukin-2 (IL-2), IL-7, IL-9, and IL-15, all of which are growth factors that use γc as a part of their receptor. Driving T or B cells to proliferate by overstimulation of growth factor pathways causes a lymphoproliferative syndrome that may increase the risk of developing aggressive malignant disease when coupled with a transforming event.

Most of the studies done in murine models have been designed primarily to evaluate efficacy of gene transfer, gene expression, and effects on disease phenotype. In light of the review of this voluminous literature, a series of recommendations were formulated to maximize the yield of relevant biosafety information that may be obtained concomitantly (Table 3).

doi:10.1016/S1525-0016(03)00212-0

LARGE ANIMAL STUDIES

Cynthia Dunbar (NHLBI, NIH) and Hans-Peter Kiem (FHCRC) assembled all available information on studies of retroviral-mediated gene transfer in large animals, both published and unpublished. Experimental and preclinical studies of gene transfer to HSCs, lymphocytes, or direct vector injection have been conducted in nonhuman primates, dogs, cats, sheep, rabbits, and pigs. These models are believed to have strong relevance to humans in that, in contrast to murine models, these larger animals not only have comparable life expectancies, similar hematopoietic demands on their HSCs, cross-reactivity of reagents (cytokine, vector envelopes, etc), but they also generally yield gene transfer efficiencies similar to those seen in clinical trials. The methods for study included PubMed identification and review of all published articles, as well as direct query to all investigators known to operate large animal facilities able to carry out gene transfer and transplantation, regarding long-term follow-up and unpublished toxicity.

There was a focused detailed analysis of cohorts with more than 1 year follow-up and more than 1% long-term levels of vector-containing peripheral blood cells (PBCs), including analysis of peripheral blood counts, gene transfer levels, and linear amplification-mediated (LAM)-PCR analysis for the number and location of proviral insertions (ongoing) to attempt to identify late-occurring adverse events. These comprehensive studies were primarily performed at the NIH on rhesus monkeys, or at FHCRC on baboons and dogs.

- 46 Rhesus macaques
  - — Median follow-up 3 years (1–6 years)
  - — Median $CD34^+$ cells infused, 82 million (9–1400 million)
  - — CBCs normal, animals healthy
  - — Clone numbers in 20 animals, median 30 (10–100)
- 21 Baboons
  - — Median follow-up 2.3 years (1–5.5 years)
  - — Median $CD34^+$ cells infused, 60 million
  - — CBCs normal, animals healthy
- 12 Dogs
  - — Median follow-up 2 years (1–4.2 years)
  - — Median $CD34^+$ cells infused, 170 million
  - — CBCs normal, animals healthy

Thus, in these extensive and well-documented studies, large animals achieving significant levels of gene marking of HSCs that were followed for up to 6 years showed no evidence for adverse events such as insertional oncogenesis. The numbers of gene-transduced cells infused are in the ranges achieved in the French XSCID study. Of note, these large animal models of retroviral-mediated gene transfer to HSCs do not have SCID or the marked selective advantage conferred upon gene-corrected cells in XSCID subjects. They also generally received vectors expressing only a marker gene, such as *neo* or GFP, and not a signaling molecule such as the γC cDNA. Thus, these results suggest that the high incidence of leukemia seen in the XSCID subjects (2/10 infants treated) is related to specific aspects of the XSCID disease state in these patients or to the specific gene being constitutively expressed; the corollary to this would be the implication that the incidence of this serious adverse event may be lower in other settings (different disease, different gene). As discussed later, review of the outcome of clinical trials with human subjects may lead to the same conclusion.

Conclusions

More than 130 dogs, 175 nonhuman primates, 49 sheep, and small numbers of pigs, rabbits, and cats have received hematopoietic cells transduced ex vivo or in vivo with integrating retroviral or lentiviral vectors. No animals exposed to helper-free vector preparations have developed leukemia, lymphoma, or any other evidence for toxicity related to the integration of gene transfer vectors.

The only evidence for toxicity resulting from retroviral gene transfer in large animals occurred in rhesus macaques transplanted with cells contaminated with RCR vectors. These animals developed T-cell lymphoma/leukemia and tumor cells had multiple helper genome insertions. It is thus far unknown whether *LMO2* is involved.

Follow-up of at least 1 year and a median of 3 years is available in a significant minority of animals that also have stable high-level long-term gene transfer levels in PBCs, including 46 rhesus macaques, 21 baboons, and 12 dogs, Thus far none have increasing levels of marked cells, monoclonality or oligoclonality, or any other worrisome features. An average of 30 clones per animal in those analyzed thus far implies that at least 3000 different insertion events most probably occurred in these animals.

- *IL2RG* gene transfer in dogs, primates, and sheep has been done. Thus far, no leukemic or clonal events have occurred, although none of the models used closely matches the human parameters in the French trial.

HUMAN CLINICAL TRIALS

Clinical trials using retroviral vectors to transfer genes to human HSCs were first conducted in 1992, and at least 40 such trials have been completed thus far. These studies have been in four areas: gene marking, chemotherapy resistance genes, anti-HIV-1 genes, and gene replacement for genetic diseases (Table 4).

Gene marking studies were done in the setting of patients undergoing autologous HSC transplantation to treat high-risk cancer or leukemia. It had been controversial whether autologous preparations of bone marrow or peripheral blood stem cells (PBSCs) were contaminated with the patient's malignant cells that could contribute to relapses after transplant. To determine whether there

TABLE 4: Summary of clinical trials performed using retroviral-mediated gene transfer to human hematopoietic stem cells

- 40 clinical trials with at least 232 subjects:
  - —7 Gene marking in autologous bone marrow transplant
    - CML (2), AML, acute leukemia, NHL, NB, breast cancer
  - — 8 Chemotherapy resistance genes
    - MDR1 (6), MGMT (2)
    - Breast (2), NHL, germ cell, carcinoma, breast/ovarian, CNS tumors, sarcoma
  - — 7 HIV/AIDS
  - 18 Genetic diseases
    - SCID: ADA (5), XSCID (2) Jak3 (1)
    - CGD (4), LAD (1), Gaucher (3), Fanconi (2)

NHL, non-Hodgkin's Lymphoma; NB, neuroblastoma.

were residual malignant cells in the transplant inoculate, the cells were exposed to a retroviral vector carrying a theoretically inert marker gene (the bacterial neomycin resistance gene); if contaminating tumor cells were present in the inocula and transduced by the vector, then there would be gene-marked tumor cells if they contributed to a subsequent relapse. This type of marking of cells at relapse was documented in studies of pediatric patients with neuroblastoma and acute myelogenous leukemia (AML), and in adults with chronic myelogenous leukemia (CML). Therefore, these marking studies successfully used retroviral vectors to achieve the scientific endpoint, although no therapeutic intent was involved. As an added element of these marking studies, the ability of the retroviral vectors to transduce normal HSCs could also be observed. In general, there was very low to undetectable transduction of normal HSCs, as suggested by the absence of gene-containing cells in marrow or blood beyond the first few months. Exceptions to this minimal marking were seen in studies with pediatric oncology patients, wherein their young age and gene transductions at the time of hematopoietic recovery after intensive chemotherapy may have produced relatively higher numbers of HSCs susceptible to retroviral-mediated transduction. No leukemia incidence or other adverse events could be attributed to the gene transfer procedure.

After the gene marking studies, there were attempts to transfer genes conferring resistance to chemotherapy to HSCs of oncology patients undergoing autologous transplant. The underlying premise was that the dose-limiting toxicity of chemotherapy is often suppression of blood cell production by bone marrow due to myelosuppression; modification of a modest percentage of HSCs with genes encoding resistance to chemotherapy could allow higher dosages of chemotherapy to be given to patients to treat resistant types of cancer. The chemotherapy resistance genes that have been studied in clinical trials include the gene encoding MDR (*MDR1*) and a gene encoding a modified form of methyl guanine methyltransferase (MGMT) that confers resistance to BCNU. As with the gene marking studies, the trials with chemotherapy resistance genes have generally yielded low levels of gene transfer into HSCs. The most successful trial, by Abonour et al., obtained up to 1% of PBCs with the transferred *MDR1* [16], with anecdotal evidence that some subjects had less severe myelosuppression from post-transplant chemotherapy than did historical control subjects. Most subjects did not have gene-containing blood cells beyond 1 year. No adverse consequences were seen from gene transfer in any of the subjects. Follow-up in many of the oncology patients in either the gene marking or chemotherapy resistance gene studies was limited by progression of the underlying malignancy. However, some of the subjects have had follow-up for as long as 10 years without adverse event from the gene transfer.

A group of studies have had the goal of transferring synthetic genes into HSCs that confer resistance to HIV-1 infection. A variety of such synthetic anti-HIV-1 genes have been developed, including those encoding antisense RNA to HIV-1, ribozymes to cleave HIV-1 RNA, dominant-negative mutants of HIV-1 regulatory genes, RNA decoys to bind HIV-1 regulatory proteins, and genes that decrease cellular receptors for HIV-1. Retroviral vectors have been developed to carry these anti-HIV-1 genes into HSCs to yield HIV-1-resistant $CD4^+$ T lymphocytes and monocytic cells. As with the other clinical trials, most of these studies achieved only low levels of gene transduction and engraftment of HSCs, with low levels of gene-containing cells seen for a few months. No adverse events attributable to the retroviral-mediated gene transfer have been seen in these studies with HIV-1-infected subjects.

The largest group of clinical trials using retroviral vectors directed to HSCs has been for genetic diseases of blood and immune cells. Studies have approached various immune deficiencies, including SCID due to defects in *ADA*, *γC* (XSCID), and Janus 3 kinase (*Jak3*) (clinically similar to XSCID, but inherited in an autosomal recessive pattern), chronic granulomatous disease (CGD), leukocyte adhesion deficiency (LAD; CD18 defect). Additionally, studies have been conducted with patients having the lysosomal storage disease Gaucher's disease and the DNA repair defect disease Fanconi's anemia. With the exception of the success in recent studies for XSCID and ADA-deficient SCID (discussed earlier), the other studies can be summarized as yielding amounts of gene transfer that were too low to be of clinical benefit.

It is in light of this earlier experience with retroviral-mediated gene transfer to HSCs, showing safety but relative ineffectiveness, that the French trial for XSCID was conducted. XSCID provides a highly favorable setting for gene transfer to HSCs to be of therapeutic benefit because

doi:10.1016/S1525-0016(03)00212-0

a strong selective survival advantage of gene-corrected T lymphocytes or their precursors would allow selective amplification of gene-corrected cells, overcoming the low efficiency of gene transfer seen in other studies. The young age of XSCID subjects (generally less than 1 year of age) may provide HSCs from bone marrow of high proliferative potential, further facilitating retroviral-mediated gene transfer. Fischer and co-workers conducted preclinical studies involving the *γC* gene knockout mouse and cell lines and primary bone marrow samples from XSCID subjects, demonstrating restoration of T and natural killer cell function without detected adverse events. The methods used for gene transfer to the subject's HSCs were those that had been developed over the decade of research in the field to yield optimal gene transfer, enriching for HSCs by isolating $CD34^+$ cells from the bone marrow and using an amphotropic-pseudotyped retroviral vector carrying the normal human γC cDNA under transcriptional control of the murine leukemia virus long terminal repeat (at relatively high titer, e.g., 10^6–10^7 cells/ml), recombinant cytokines *flt*-3 ligand, c-*KIT* ligand, thrombopoietin, and IL-3 to stimulate HSC proliferation, and recombinant fibronectin. The extent of gene transfer, measured by FACS analysis of transduced $CD34^+$ cells and by analysis of myeloid colonies grown from the transduced marrow (CFU-C), were in the range typically achieved with these methods, 25–50%. The only remarkable finding from the treatment data, currently of uncertain significance, was that relatively high numbers of bone marrow $CD34^+$ cells were obtained from the subjects, in the range of 10×10^6 to 30×10^6 cells/kg. Although the absolute numbers of $CD34^+$ cells given to the infants were not excessive compared with those given to adult subjects, especially when using G-CSF mobilized PBSCs, they were high on a per-kilogram basis. If insertional oncogenesis is a purely stochastic event, then the risk of transformation should be proportional to the absolute number of transduced cells given to subjects. However, the relatively high numbers of $CD34^+$ cells per kg obtained from the XSCID infants suggests that there may be some disease- or age-specific factors that led to increased numbers of stem or progenitor cells that were susceptible to transduction. For example, the defect of the γC cytokine receptor may have led to a maturation block in lymphoid progenitor cells, expanding the pool of these cells that could be transduced and engrafted, increasing the stochastic risk. Alternatively, the strong selective survival/expansion advantage for cell expressing a normal *γC* gene may have led to exceptionally high engraftment and expansion of transduced cells

The long latency between gene transfer and the development of clinically detected leukemia was more than 2 years on both subjects. There remain a number of subjects in the French and British studies for XSCID and the Italian study for ADA deficiency who had their gene treatment more recently, and obviously these cohorts of patients will require close clinical follow-up.

SUMMARY

In summary, this review of findings in murine and large animal models and in human clinical trials indicates that the development of leukemia in the XSCID subjects was relatively unprecedented and not predicted by preclinical studies. No SAEs related to retroviral vectors were observed, except for a single report in a murine model and the two cases in the XSCID trial. However, in contrast to the very high number of gene-containing cells seen in the XSCID studies (and the Italian trial for ADA deficiency), most of the other clinical studies have been characterized by very low numbers of gene-containing cells in circulation (<1–2 years) and a lack of long-term persistence. Many subjects in the gene marking studies were patients with advanced malignant disease (undergoing therapeutic hematopoietic stem cell transplantation) to which they succumbed within months to a few years of participation in the gene transfer trial. Most treatments for genetic diseases were done without any cytoreductive therapy, and thus engraftment of gene-containing cells was in very low numbers. Thus, the lack of SAEs in these studies cannot be used to support the general safety of retroviral-mediated gene transfer to HSCs. One cannot make conclusions about the safety of gene therapy when there is not significant gene transfer to the relevant target cells. It is possible that the amount of gene transfer to HSCs in these studies was higher than detected by gene marking of PBCs, and the absence of lymphoproliferation indicates that this is not necessarily a problem. The few exceptional studies that did show more long-term persistence of gene-marked cells did not have any cases of leukemia or other complications.

RECOMMENDATIONS

A series of specific recommendations to improve murine gene transfer studies to yield useful toxicology data can be found in Table 3. Similar efforts to obtain both safety as well as efficacy data from large animal studies are indicated. For clinical trials, it would be crucial to maintain ongoing databases to allow cross-comparisons of study outcomes. The NIH RAC is developing a database of all clinical trials of gene transfer conducted in the United States, and a similar effort is encouraged in other countries. The International Bone Marrow Transplant Registry (IBMTR) has been accruing exactly this type of data for HSC transplants and has the relevant expertise in database management, biostatistics, and bioethics to undertake this effort to catalog clinical trials of gene transfer to HSCs. IBMTR has formed a working group to establish and maintain a database on clinical trials involving gene transfer to HSCs.

The US Food and Drug Administration has placed on hold all clinical trials using retroviral vectors to transduce HSCs and will require the development of plans to mon-

itor subjects for the emergence of monoclonal cell proliferations. The costs for these mandated investigations will need to be provided for academic investigators. Specifically, it will be important to support:

- Cores for clinical trial monitoring tests. Long-term observation of experimental animals for late events, especially for large animals. Studies specifically focused on analyses of risks from integrating vectors, the functions of specific transgene products, stem cells in different disease states, etc. Development of safer methods of gene transfer methods, such as nonintegrating but persisting vectors, site-specific integration, insulators, suicide genes.

The information gathered here represents the voluntary effort of independent members of ASGT and does not constitute "official" opinions of the ASGT. Writers worked from available published literature and data provided by investigators. The intent was to be complete and accurate, but some relevant studies may have been inadvertently overlooked or misquoted. Any suggested additions or corrections would be welcome and may be submitted via the ASGT website (www.asgt.org).

REFERENCES

1. Rosenberg, S. A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R. A., Moen, R., Karson, E. M., Lotze, M. T., Yang, J. C., Topalian, S. L., *et al.* (1990). Gene transfer into humans-immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N. Engl. J. Med.* **323:** 570–578 (see comments).
2. D. B.Kohn, G. M.Crooks, Clinical trials of gene therapy using hematopoietic stem cells. In: E.D. Thomas, K.G. Blume, S.J. Forman (Eds.), Hematopoietic Cell Transplantation, 3rd edition, Blackwell Scientific Publications, Boston, 2003, pp. xx–xx.
3. Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J. L., Bousso, P., Le Deist, F., and Fischer, A. (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288:** 669–672.
4. Hacein-Bey-Abina, S., Le Deist, F., Carlier, F., Bouneaud, C., Hue, C., De Villartay, J. P., Thrasher, A. J., Wulffraat, N., Sorensen, R., Dupuis-Girod, S., Fischer, A., Davies, E. G., Kuis, W., Leiva, L., and Cavazzana-Calvo, M. (2002). Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N. Engl. J. Med.* **346:** 1185–1193.
5. Aiuti, A., Slavin, S., Akerm, M., Ficara, F., Deola, S., Mortellaro, A., Morecki, S., Andolfi, G., Tabucchi, A., Carlucci, F., Marinello, E., Cattaneo, F., Vai, S., Servida, P., Miniero, R., Roncarolo, M. G., and Bordignon, C. (2002). Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* ***296:*** 2410–2413.
6. Hacein-Bey-Abina, S., von Kalle, C., Schmidt, M., Le Deist, F., Wulffraat, N., McIntyrel. Radford, E., Villeval, J. L., Fraser, C. C., Cavazzana-Calvo, M., and Fischer, A. (2003). A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.* **348:** 255–256.
7. Belmont, J. W., *et al.* (1988). Expression of human adenosine deaminase in murine hematopoietic cells. *Mol. Cell Biol.* **8:** 5116–5125.
8. Li, Z., *et al.* (2002). Murine leukemia induced by retroviral gene marking. *Science* ***296:*** 497.
9. Sadelain, M., and Riviere, I. (2002). Sturm und Drang over suicidal lymphocytes. *Mol. Ther.* **5:** 655–657.
10. Villeval, J. L., *et al.* (1997). High thrombopoietin production by hematopoietic cells induces a fatal myeloproliferative syndrome in mice. *Blood* **90:** 4369–4383.
11. Innes, K. M., *et al.* (1999). Retroviral transduction of enriched hematopoietic stem cells allows lifelong Bcl-2 expression in multiple lineages but does not perturb hematopoiesis. *Exp. Hematol.* **27:** 75–87.
12. Li, Z., *et al.* (2002). Persisting multilineage transgene expression in the clonal progeny of a hematopoietic stem cell. *Leukemia* **16:** 1655–1663.
13. Bunting, K. D., Galipeau, J., Topham, D., Benaim, E., and Sorrentino, B. P. (1998). Transduction of murine bone marrow cells with an MDR1 vector enables ex vivo stem cell expansion, but these expanded grafts cause a myeloproliferative syndrome in transplanted mice. *Blood* **92:** *2269–2279*.
14. Bunting, K. D., Zhou, S., Lu, T., and Sorrentino, B. P. (2000). Enforced P-glycoprotein pump function in murine bone marrow cells results in expansion of side population stem cells in vitro and repopulating cells in vivo. *Blood* ***96:*** 902–909.
15. Licht, T., Goldenberg, S. K., Vieira, W. D., Gottesman, M. M., and Pastan, I. (2000). Drug selection of MDR1-transduced hematopoietic cells ex vivo increases transgene expression and chemoresistance in reconstituted bone marrow in mice. *Gene Ther.* **7:** 348–358.
16. Abonour, R., Williams, D. A., Einhorn, L., Hall, K. M., Chen, J., Coffman, J., Traycoff, C. M., Bank, A., Kato, I., Ward, M., Williams, S. D., Hromas, R., Robertson, M. J., Smith, F. O., Woo, D., Mills, B., Srour, E. F., and Cornetta, K. (2000). Efficient retrovirus-mediated transfer of the multidrug resistance 1 gene into autologous human long-term repopulating hematopoietic stem cells. *Nat. Med.* ***6:*** 652–658 (Comment in Nat. Med. 6 (2000), 624–626).