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Getting Human Gene Therapy to Work

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Human gene therapy began September 14, 1990, when a 4-year-old girl with adenosine deaminase (ADA) deficiency received an infusion of autologous T cells into which a normal ADA gene had been inserted. The technical approaches for gene transfer had been well-established over many years; therefore, this event represented not necessarily a scientific achievement, but a cultural breakthrough in its application to a human subject [1]. Following treatment, this young girl's T-cell number normalized and the frequency of her infections decreased. Unfortunately, since the ADA gene was inserted into mature T cells and not pluripotent stem cells, repetitive therapies were required in a second patient. Introducing genes into early stem cells to accomplish perpetual gene replication and expression is one of the most challenging problems facing investigators in the field of human gene therapy.

The Recombinant DNA Advisory Committee (RAC) has approved more than 60 protocols for human gene therapy. Table I provides several representative examples. Criteria have been proposed as guidelines for applying the technology of gene therapy to humans. Candidate diseases include serious life-threatening illnesses for which current therapy is inadequate. The gene in question should have been isolated, cloned, and characterized. Genes with a simple regulation scheme and those that code for a single-chain protein are ideal. The transfer of a normal gene and its control elements to supplement a defective gene that fails to produce a deficient protein or that produces an inactive or nontoxic product may be successful, even though the retroviral vector carrying the gene complex integrates randomly into the host genome. If the defective gene produces a product that is toxic to host cells, corrective gene transfer strategies may be required, e.g., "gene knockout." These methods are technically difficult and may require site specific insertion to inactivate the pathogenic gene.

With current technology and safety requirements, ex vivo access to defective tissues is important. In this regard, much work has focused on the genetic manipulation of bone marrow or blood cells ex vivo. Somatic cell targets such as bone marrow are the focus of current human gene therapy protocols. With continued progress and a focus directed toward new frontiers, human transgenic therapy may be possible. Major ethical issues must be resolved before genes are inserted into fertilized eggs, as this approach potentially alters the genetic makeup of future generations.

RETROVIRAL-MEDIATED GENE TRANSFER

There are a number of methods for introducing genes into cells that involve the use of both physical techniques and/or viral vectors. Most gene therapy approaches have used retroviral vectors [2–6] targeted to selected cell populations. These vectors have a high efficiency of gene transfer into replicating cells, integrate directly into cellular DNA, and have been engineered so that they cannot self-propagate. Retroviruses are able to entertain more foreign genetic material than some DNA viruses, infect a broad spectrum of cell types and species, and have a high level of expression of internal sequences. Only single-copy inserts are incorporated into the host genome, minimizing the risk of insertional mutagenesis. Retroviruses enter primate and human cells by interaction with cell surface molecules. However, these amphotropic receptors are found infrequently on hematopoietic stem cells, a potential disadvantage for accomplishing long-term gene expression. Retroviral vectors are produced at relatively low titer and only integrate genetic material in cycling cells (most stem cells are in noncycling state). Integration into the host genome is random and,

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TABLE I. Selected Human Gene Therapy Clinical Protocols Approved by the Recombinant DNA Advisory Committee

P.I.	Title	Summary
Blaese RM	Treatment of Severe Combined Immune Deficiency (SCID) Due to Adenosine Deaminase (ADA) Deficiency With Autologous Lymphocytes Transduced With a Human ADA Gene	ADA gene product restores T-cell number and function
Rosenberg SA	Gene Therapy of Patients With Advanced Cancer Using Tumor Infiltrating Lymphocytes (TIL) Transduced With the Gene Coding for Tumor Necrosis Factor	TIL cells adhere to tumors; TIL cells transduced with TNF gene may kill cancer cells
Wilson JM	Ex Vivo Gene Therapy of Familial Hypercholesterolemia	Congenital lack of LDL receptor restored after ex vivo treatment of liver with vector containing LDL receptor gene; cholesterol in LDL then lowered in serum
Oldfield E	Gene Therapy for the Treatment of Brain Tumors Using Intra-Tumoral Transduction With the Thymidine Kinase (TK) Gene and Intravenous Ganciclovir	TK gene introduced into tumor cells; Ganciclorin administered in vivo causes accumulation of toxic metabolite in TK transduced cells; neighboring cells also affected ("innocent bystander") effect correction of cystic fibrosis cellular defect in Cl^- secretion
Crystal RG	Gene Therapy of the Respiratory Manifestations of Cystic Fibrosis Using a Replication Deficient, Recombinant Adenovirus to Transfer the Normal Human Cystic Fibrosis Transmembrane Conductance Regulator cDNA to the Airway Epithelium	
Karlsson S	Retroviral-Mediated Transfer of the cDNA for Human Glucocerebrosidase Into Hematopoietic Stem Cells of Patients With Gaucher Disease	Correction of enzyme defect; no myeloblation
O'Shaughnessy J	Retroviral-Mediated Transfer of the Human Multi-Drug Resistance Gene (MDR-1) Into Hematopoietic Stem Cells During Autologous Transplantation After Intensive Chemotherapy for Breast Cancer	MDR1 gene inserted into "normal" bone marrow from breast cancer patients; after transplants, patients who relapse receive Taxol; evaluate ability of MDR1 to protect marrow from myelosuppressive effects of Taxol
Hsueh JL	Treatment of Hemophilia B With Autologous Skin Fibroblasts Transduced With a Human Clotting Factor IX cDNA	Skin fibroblasts transduced with factor IX gene and reimplanted for hemophiliacs
Nabel A	HLA-B-7 Into Cancer Cells	Gene for HLA B7 introduced into cancer cells that are reimplanted triggering general immune response against tumor
Brenner M	Treatment of Neuroblastoma With IL-2 Transfected Cancer Cells	IL-2 gene introduced into neuroblastoma cells that are reimplanted and augment immune response against tumor

theoretically, may cause insertional mutagenesis leading to cancer.

Typical retroviral vectors have long terminal repeats (for viral integration into the host genome) and the sequences for replication of viral internal structural protein, enzymatic proteins, and envelope glycoproteins contained between the LTRs are deleted and replaced by the desired gene [4,7,8]. In order to construct infectious viral particles with the inserted gene, the proteins need to be supplied. They can be provided by the presence of a replication competent helper virus. Packaging cell lines with a defective virus genome, but capable of producing the necessary proteins required for virion assembly, have been developed to obtain vector preparations free of helper virus [9]. These lines cannot package their own genetic material because the encapsidation sequence (ψ sequence) required for sufficient packaging of the viral RNA genome has been deleted [2,8,10]. When a retroviral vector (which contains the encapsidation sequence) is transferred into a packaging cell, selective packaging of the vector RNA occurs. Thus, this producer cell assembles a vector free of replicating virus. However, producer cells can become a source of replication competent virus if a recombination event occurs in which the vector transfers its ψ sequence to the defective encapsidation sequence-negative virus [6,7,9,11–14]. This runs the risk of direct biologic effects by the helper virus, such as the recently documented development of helper virus-induced lymphomas in primates [15,16]. In typical human retroviral gene therapy approaches, the supernatant of the packaging cells containing retroviral particles is harvested and incubated with target cell populations for DNA integration. Alternatively, in nonhuman systems, target cells are co-cultured with the producer cells, usually in the presence of a cytokine cocktail. Retroviral vectors integrate directly into cellular DNA after reverse transcription of their RNA genome. There is the theoretical risk of insertional mutageneses with these techniques, but most animal models have not confirmed this possibility [17], in part probably because disruption of a single proto-oncogene is unlikely to lead to malignant transformation [6,17–24].

CELLULAR APPROACHES TO IMPROVE HUMAN GENE THERAPY

A number of obstacles must be surmounted before these treatments achieve success in humans. A major limitation is a failure to achieve adequate gene transfer efficiencies into primitive hematopoietic stem cells and an inability to expand these cells in vivo. The long-term expression of human genes in vivo after transplant will be influenced by (1) the efficacy of gene insertion into long-term renewal cells, (2) the ability of the cells to engraft in vivo, and (3) the ability to express the gene in primitive cells and their progeny.

Developmental Gene Delivery Systems

A variety of gene delivery systems other than retroviruses are being explored to improve gene transfer and expression. Herpes simplex virus (HSV) has the capacity to carry more than 10 kb of DNA and has a tropism for neural tissue. This vector has been used to deliver the viral thymidine kinase gene, which, after exposure to ganciclovir, produces a product toxic to brain tumor cells. Similarly, the adenovirus has a tropism for respiratory epithelium and may be a suitable vehicle in aerosolized form to correct the genetic abnormality in young patients with cystic fibrosis. However, most DNA vectors transfer DNA episomally, and gene replication and expression in progeny cells are not sustained. "Hybrid" vectors that combine advantages of several current vectors or new vectors, e.g., safety-modified versions of the human immunodeficiency virus (HIV), may offer an attractive new classes that are highly efficient. Liposomes or artificial chromosomes may allow the transfer of large amounts of DNA to target cells. Full levels of physiologically controlled expression can be obtained by transfer of intact genes on artificial chromosomes. These particles contain a centromere and telomeres to allow maintenance and segregation in mammalian cells. Liposomes or receptor-mediated transfer may permit delivery of these large DNA fragments. Appropriately engineered cellular systems may eventually permit the infusion or implantation of producer cell lines directly into patients to maximize gene transfer efficiency. Modulation of the host's immune response to these approaches may play a significant role in the success of various gene delivery systems.

In clinical applications of gene therapy, the protein must be expressed to achieve the desired clinical effect. Expression of a transgene is dependent upon transcription initiated by the retroviral promoter located on the viral LTR. When a replacement gene is coupled with a dominant,

selectable marker, e.g., neomycin resistance gene (NEO^R), an alternative vector design may be used. This design could include an independent promoter to transcribe the second gene in either "sense" or "antisense" configuration [25]. Such a "two-gene" vector may be associated with diminished gene expression.

If gene therapy is applied to complex diseases such as thalassemia, an understanding of vector-associated promoters, enhancers, introns, and LCR elements is needed to correctly control transgene expression [26]. Although promoter and enhancer regions drive gene expression by binding to a variety of general and tissue-specific transcription factors, the levels of expression varies depending on the integration site into the host genome. From studies of human β-globin in transgenic mice, critical regions located on either site of the gene cluster must be linked and included in the gene delivery construct. With this approach, full levels of expression may be restored regardless of the integration site. Yeast artificial chromosomes (YACS) have ample capacity to carry complete human genes with associated control elements, even when these are located within the introns of the genes or at a distance upstream or downstream from the gene [26]. These methods provide promising approaches for correcting complex genetic diseases.

Stem Cell Purification and Cytokines

Genes have been successfully inserted into pluripotent stem/progenitor cells in mice, dogs, and primates [5,27–31]. Selection of hematopoietic cell populations likely to contain replicating or early cells, e.g., $CD34^+$, umbilical cord, long-term culture initiating cells (LTCIC) and fetal cells, may increase gene insertion frequency and the capacity for long-term expression [28,32]. Genes have been expressed in myeloid and lymphoid lineages for more than 2 years and after serial transplants indicating the likelihood of transduction into multipotent marrow renewal cells [29,30]. Similar observations have been noted in vivo and in vitro with human cells [27,33]. Unfortunately, the percentage of cells maintaining long-term expression in general is relatively low (0.1–5%), and their detection usually requires the use of polymerase chain reaction (PCR) assays and/or growth of hemopoietic colonies with a neomycin resistance selection marker in neomycin [3].

A critical feature of marrow-based gene therapy approaches is the use of selected cytokines to maintain and/or induce cycling of primitive marrow stem cells in in vitro culture, or when given to the donor animal [2,3,34–37]. In addition, the feasibility both of in vitro gene insertion into stem cells and in vivo cell administration clearly would be increased if appropriate long-term renewal cells could be purified. The use of purified stem cells would decrease total retroviral titer needed and could give superior engraftment (not established). Alternatively, in transplant studies removal of accessory cells may lead to poor engraftment. Many groups have shown that early marrow stem/progenitor cells, mirrored by the blast colony-forming cell, the HPP-CFC and the long-term culture initiating cell (LTC-IC) are responsive to multiple cytokines. The combinations of cytokines, especially those including Steel factor, are most effective in supporting these cells [31,38–48], although in most cases the balance between survival, proliferative, and differentiative effects has not been defined. Cytokines that appear to selectively support early stem cells include Steel factor, IL-11, IL-1α, IL-6, and IL-3, but evolving studies suggest possible roles for leukemic inhibitory factor (LIF) [49], IL-12 [50], IL-13 [51], and basic fibroblast growth factor (bFGF) [52–54]. Exposure of marrow cells to GM-CSF and IL-3 [55] or IL-3, IL-6, and Steel factor [56] has also been shown to enhance overall engraftment of transplanted marrow cells.

A number of inhibitors of stem cells have been reported, some of which have a nontoxic reversible action on cycling primitive marrow stem cells [57]. These include macrophage inflammatory protein-1α [58,59], which appears to block the entry of cells into S and removes cells from S-phase, a tetrapeptide (acetyl-*N*-Ser-Asp-Lys-Pro), which blocks the entry of stem cells into S [60] and a pentapeptide (pGlu-Glu-Asp-Cys-Lys), that removes stems from S-phase [61]. These agents have not yet been tested for their effect on retroviral integration and in vivo engraftment of cycling stem cells.

Although many approaches to human stem cell purification have been reported, there is still no consensus on the true nature of the long-term lymphohematopoietic marrow renewal cell. In general, marrow cells are depleted of lineage specific differentiated cells using specific monoclonal antibodies (mAb) and immunomagnetic or panning separative approaches, and cells are

then selected for specific antigens that appear to be present on early stem cells. The most effective separations appear to have been obtained using mAbs selecting for CD34 and the c-*kit* receptor. The removal of more mature progenitor cells using antibodies to CD38 and HLA-DR antigens have provided a means to enrich significantly for early cell populations. Rhodamine staining has also been used for subsetting marrow repopulating cells. Finally, the combination of low Hoechst and rhodamine staining may provide a powerful and relatively easy means of purifying early marrow renewal cells.

The development of stromal cell layers in conjunction with short-term conventional methods of cell culture appears to enhance retrovirus-mediated gene transduction into human cells [62]. With this method, direct cell–cell interactions between stroma and adherent cells or local elaboration of cytokines in gradient concentrations may play a role in either sustaining stem cell growth or increasing the number of cycling stem cells, rendering them more susceptible to retroviral transduction.

The ability to expand early progenitors and stem cells in culture will obviously lead to profound improvements in our ability to deliver effective gene therapy. Berenson and coworkers are investigating the effects of prolonged ex vivo suspension culture of CD34-enriched marrow cells and have found that IL-3, IL-6, SCF, and IL-1 greatly expand the number of both late progenitors (CFU-GM) and earlier progenitors (HPP-CFC) present after 1–3 weeks of culture [63].

All together, the optimum stem cell purification/cytokine conditions facilitating gene transfer/engraftment have probably not yet been defined; the optimum cell phenotypes, specific cytokine(s), their correct concentrations, and the best sequence of exposure continue to be studied.

Engraftment of Genetically Transduced Cells

Current gene therapy approaches routinely include intensive myelosuppression to "open" marrow "spaces" and permit transduced cells to predominate in the host. This remains a standard approach to enhance the growth of genetically manipulated cell populations. However, in the murine system we have developed data that transplantation of autologous marrow into nonmyeloablated recipients leads to long-term chimerism, frequently exceeding 50% when male marrow cells are infused into female recipients [64]. If successful in humans this approach could minimize the toxicity of myeloablative therapy used to enhance engraftment and dominance of transduced marrow.

Although this concept was initially explored by Brecher et al. [65], we recently reported the successful long-term engraftment of normal male donor bone marrow transfused into nonablated female mice, challenging the assumption that 'niches' need to be created for marrow to engraft. Balb/c, BDF1, or CBA-J female hosts (no irradiation) received 40×10^6 male marrow cells of the same strain daily. To evaluate repopulation patterns, we used chromosomal banding and Southern blot analysis using the Y-chromosome-specific pY-2 probe [66]. The percentage of male DNA was calculated on the basis of densitometry comparisons between post-transplant DNA samples and male murine DNA. Percentages of male engrafted cells ranged from 23% to 78% (mean 38%) for recipients of normal donor marrow. At ten to twelve months the mean engraftment for the normal donor group was 46%.

Marrow transplantation into nonmyeloablated human hosts has not been attempted. However one provocative case report suggests that this may be feasible. In a recent report by Collins et al. [67], a liver transplant recipient showed donor derived long-term multilineage hematopoiesis. On day 39, post-transplantation RFLP analysis of peripheral blood cells in this patient showed that 90–95% of DNA was derived from donor and only 5–10% was derived from the recipient. Subsequent RFLP analyses of peripheral blood on days 85 and 113 and bone marrow cells on day 120 showed at least 95% of the DNA was derived from the donor. This patient had graft-versus-host disease (GVHD) and presumably died from this complication on day 135. While this situation is different from autologous transplantation, and it might be speculated that GVHD actually augmented engraftment, these data altogether suggest that the nonmyeloablated human host may be repopulated by a relatively small number of engrafting human hematopoietic cells.

Linkage to Genes Conferring a Selective Growth Advantage

Amplification of genetically transduced cellular populations may be possible by linking the transfer gene in question to a gene that confers

a selective growth or survival advantage to the cells. The *MDR1* gene encodes a 170-kd molecular-weight plasma membrane protein, termed P-glycoprotein, that acts as a drug/toxin efflux pump, extruding various chemotherapeutic agents such as Taxol, doxorubicin, and vinblastine, thereby rendering cells expressing this gene drug resistant [68,69]. This protein is widely expressed on the surfaces of epithelial cells of the intestine, liver, and pancreas, in the kidney, adrenal cortex, placenta, and in capillary endothelial cells in the testes and brain. It is also expressed in primitive hematopoietic stem cells [70]. P-Glycoprotein functions as a multidrug transport protein that extrudes hydrophobic compounds from cells. Sensitive cells become resistant to drugs that are recognized and expelled by the transporter when transduced with the *MDR1* gene. Transgenic mice with *MDR1* DNA were rendered resistant to the myelosuppressive effects of certain chemotherapeutic agents and transplantation of marrow from these transgenic animals protected mice from the myelosuppressive effects of appropriate chemotherapeutic drugs [71,72]. In some studies, expression of *MDR*-containing progenitors has increased from less than 5% to nearly 100% in mice treated for several months with Taxol.

In animal model systems, transplantation of bone marrow cells expressing the human *MDR1* gene into ablated mice results in long-term protection against the myelosuppressive effect of chemotherapy in mice. Sorrentino et al. [73] used a retroviral vector containing the human *MDR1* cDNA promoted by the Harvey murine sarcoma virus long terminal repeats to generate a high-titer, helper virus-free, ecotropic producer clone from the GP + 86 [74] packaging cell line. Bone marrow cells from donor mice pretreated with 5-fluorouracil (5-FU) to induce stem cell cycling were co-cultured with producer cells in the presence of murine IL-3 and IL-6 and then used to reconstitute W/W^v mice. Mice were treated with a single intraperitoneal bolus of Taxol. Although *MDR1* was detectable in animals at 11 weeks after transplant, 6 months after transplant, no provirus was detected in cell samples obtained from the animals. Animals given a another dose of Taxol developed severe myelosuppression. Thirteen days later, after recovery of counts, circulating leukocytes appeared to have detectable provirus. When the animals were rechallenged with Taxol on day 17, the average neutrophil count dropped only 7%, compared to 42% for the NEO^R marked controls [73]. Other investigators have developed similar models in murine or canine systems [75–77].

These approaches attest to the feasibility of gene transfer with *MDR1* cDNA into murine or human cells and offer the possibility for therapeutic applications. *MDR1* inserted into normal marrow from cancer patients may reduce the myelosuppressive toxicity and/or permit the delivery of higher, more therapeutic, doses of drugs to cancer patients.

Deisseroth et al. [77] recently received approval by the Recombinant DNA advisory committee to proceed with human gene therapy experiments in ovarian cancer patients using *MDR1* transduced marrow and render it resistant to Taxol chemotherapy. Similar protocols for patients with breast carcinoma have been proposed. These approaches are a first step to link *MDR1* with other genes in an effort to expand transduced populations of cells.

The future of gene therapy as a treatment in medicine depends on a continued series of incremental successes in molecular medicine and cell biology. With these steps, the keys that unlock the human genome and open it to improvement and modification will be forthcoming.

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