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STEM CELL DIRECTED GENE THERAPY

Barbara C. Engel and Donald B. Kohn

Division of Research Immunology/Bone Marrow Transplantation, Childrens Hospital Los Angeles, Department of Pediatrics, University of Southern California, School of Medicine

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1. ABSTRACT

A potential therapeutic approach to HIV-1 infection is the genetic modification of cells of a patient to make them resistant to HIV-1. Hematopoietic stem cells are an attractive target for gene therapy of AIDS because of their ability to generate a broad repertoire of mature T lymphocytes, as well as the monocytic cells (macrophages, dendritic cells and microglia) which are also involved in HIV-1 pathogenesis. A number of synthetic "anti-HIV-1 genes" have been developed which inhibit HIV-1 replication. However, current methods for gene transfer into human hematopoietic stem cells, using retroviral vectors derived from the Moloney murine leukemia virus, have been minimally effective. Clinical trials performed to date in which hematopoietic cells from HIV-1-positive patients have been transduced with retroviral vectors and then reinfused have produced low to undetectable levels of gene-containing peripheral blood leukocytes. New vector delivery systems, such as lentiviral vectors, need to be developed to ensure efficient gene transfer and persistent transgene expression to provide life-long resistance to the cells targeted by HIV-1.

2. INTRODUCTION

Although the recent breakthroughs in anti-viral chemotherapy have produced great benefits to many people infected with human immunodeficiency virus-1(HIV-1) (1-3), there is still room for additional, complementary modalities to try to sustain immune function and health. As the field of gene therapy has developed, applications to treatment of infectious diseases, such as HIV-1 infection, have been explored (4-9). The question about the optimal target for gene therapy for HIV has not yet been resolved. Initial clinical trials used $CD4^+$ T lymphocytes (7) which are relatively easy to obtain, isolate and transduce. As mature T cells have a limited life span, in the long run, repeated transfers of gene-modified cells would be required.

In addition to CD4 + T lymphocytes, other cells like T cell precursors in the thymus and lymph nodes as well as monocytes/macrophages, dendritic cells and microglia in the brain are also infected during the course of the acquired immunodeficiency syndrome (AIDS). Hematopoietic stem cells (HSC) give rise to the full spectrum of cells involved in AIDS pathogenesis (10, 11). Therefore, the hematopoietic stem cells contained in either bone marrow, peripheral blood or the umbilical cord from newborns represent a logical target cell for gene therapy of AIDS. HSC are long-lived, producing new progeny cells for the life of the recipient after transplant. Theoretically, insertion of a gene capable of conferring resistance to HIV-1 into hematopoietic stem cells would result in that gene being present in the descendant mature T lymphocytes and other HIV-1 susceptible cells.

Despite more than 15 years of research in the field of gene therapy using hematopoietic stem cells, the major hurdle remains the inability to efficiently and stably insert genes into these cells. Retroviral vectors based upon the Moloney murine leukemia virus (MLV) have been used most extensively, but yield relatively low gene transfer into pluripotent human HSC and gene expression which is often unsatisfactory.

3. PHENOTYPE OF HEMATOPOIETIC STEM CELLS

Stem cells as well as lineage-committed hematopoietic progenitor cells share the sialomucin CD34

as an essential marker (12), that is downregulated as the cells differentiate into more mature cells. About 1-4% of cells in bone marrow and <0.2% of cells in unmobilized peripheral blood are CD34-positive (13). The more primitive stem cells lack the differentiation antigens expressed on lineage-committed progenitor cells and are hence $CD38^-$, $CD45^{lo}$, and $HLA\text{-}DR^-$. All human fetal and neonatal hematopoietic stem cells express the Thy-1 antigen, which is only present on 5-25% of adult lineage-committed progenitors (14-16).

To date, most clinical and experimental protocols involving HSC utilize $CD34^+$-selected cells, the subpopulation with the highest multilineage potential still being under investigation. (There is recent evidence that a CD34-negative cell fraction of the human hematopoietic system can repopulate the bone marrow in a murine model; 17).

4. SOURCES OF HEMATOPOIETIC STEM CELLS

Hematopoietic stem cells can be obtained from allogeneic as well as autologous sources. Allogeneic stem cell sources have the disadvantage of potentially inducing a graft-versus-host (GvH) disease from contaminating donor T cells, or of being rejected by the recipient's immune system, but they do not bear the risk of HIV-1 contamination. Autologous cells do not raise the immunological problems of GvH or rejection, but may contain cells bearing HIV.

The most commonly used source of HSC is peripheral blood hematopoietic stem cells (PBSC), which have largely replaced bone marrow in the setting of autologous transplantation (18, 19). PBSC are mobilized from the bone marrow into the peripheral circulation by administration of factors such as G-CSF or GM-CSF for 3-5 days and can then be collected by leukapheresis. Several studies have shown that engraftment occurs faster when transplanting peripheral blood stem cells compared to bone marrow (20, 21). The clonogenic progenitor cells contained in G-CSF-mobilized PBSC are quite susceptible to retroviral-mediated gene transfer, whereas the transduction rate of long-term reconstituting stem cells in PBSC is no better than bone marrow (22-24). It has been shown that HIV-1 infected subjects can have successful mobilization and collection of G-CSF-mobilized PBSC without any increase in endogenous HIV-1 levels, at least during early stages of disease (25, 26).

Another source of hematopoietic stem cells is umbilical cord blood (UCB) which has been shown to be susceptible to retroviral transduction, potentially even more so than bone marrow cells (27, 28). Use of UCB cells HSC could be particularly beneficial for HIV-1 infected neonates. Since transmission is mostly perinatal, the umbilical cord blood should contain normal numbers and function of hematopoietic stem cells, which may be diminished in the bone marrow of HIV-1 infected children and adults (29).

5. ANTI-HIV-1 GENES

A large number of synthetic genes have been developed which can suppress HIV-1 replication ("anti-HIV-1 genes"), including: antisense, ribozymes, dominant-negative mutants (e.g. RevM10), RNA decoys, intracellular antibodies to prevent expression of viral proteins or cellular co-receptors, etc. (30-38). In many cases, these anti-HIV-1 genes have been shown in model systems to be able to significantly suppress the replication of HIV-1 and in some cases even limit virus entry into cells (36, 39-44). If essentially 100% of a patient's HSC and the resultant T lymphocytes and monocytic cells could be made incapable of supporting HIV-1 replication, it is likely that decreased viral burdens would result. Theoretically, active inhibition of HIV-1 replication in 99.9% of the susceptible cells would be required to produce a 3-log reduction in virus load, an effect often produced by highly-effective anti-retroviral therapy. However, with the limited capabilities to effectively transduce high percentages of human hematopoietic stem cells, it is not currently possible to protect the majority of susceptible cells. An alternative mechanism for efficacy is based on the possibility that cells engineered to be incapable of supporting active HIV-1 replication may be protected from viral-induced cytopathicity and thus have a selective survival advantage compared to non-protected cells. In that case, a modest number of protected cells may comprise an increased percentage of all T lymphocytes, leading to some preservation of immune function.

6. GENE TRANSFER INTO HEMATOPOIETIC STEM CELLS

6.1. Retroviral vectors

The best characterized and - because of their efficiency, safety and stable integration - mostly used delivery vehicles for preclinical and clinical gene therapy studies are murine retroviral vectors. However, there are limitations to their use, especially in hematopoietic stem cell transduction: they require cell division for their integration and the titers of recombinant vectors commonly achieved are relatively low (45, 46). Recent approaches to pseudotype the standard MoMuLV-based vectors with the envelope proteins of either the Gibbon Ape leukemia virus (47), or the G protein from vesicular stomatitis virus (48) resulted in stable viruses achieving greater titers. Additional improvements have been made in the transduction conditions, using recombinant fibronectin support, new cytokines (Flt-3 ligand, thrombopoietin), and manipulation of cell cycle kinetics (49-53). Combinations of these techniques have resulted in modest, yet significant, increases in gene marking in primate stem cell transplant models (e.g. 10-20% up from the previous ceiling of 0.1-1.0%; 54, 55). However, even higher levels of gene transduction of stem cells are likely to be needed for applications to many genetic diseases and AIDS.

6.2. Lentiviral vectors

More recently developed vector systems with promise as vehicles for stably transducing quiescent hematopoietic stem cells are based on lentiviruses, such as HIV-1. Lentiviral vectors have been shown to be able to transduce quiescent cells such as neurons, hepatocytes, and others (56).

Earlier attempts to develop vectors based on HIV-1 showed limited results, with low titers (57, 58). More recently, HIV-1-based vectors have been produced with higher transduction capacity, primarily from applying more recently obtained knowledge of HIV-1 virology and from using VSV-G protein pseudotyping which produces virion with sufficient physical stability to allow concentration to high titers by ultracentrifugation (56, 59). Studies examining the ability of these lentiviral vectors to transduce primitive human hematopoietic progenitor cells have shown great promise. Whereas MLV-based vectors can only transduce mature lineage-committed progenitor cells following prolonged stimulation with recombinant cytokines, lentiviral vectors have been shown to be capable of transducing the primitive progenitor cells which engraft in immune-deficient NOD-SCID mice (60). They also transduce quiescent $CD34^+CD38^-$ progenitors which will grow in extended long-term initiating cell culture (61), and can do so following a single exposure of cells to vectors on the day of isolation.

While there are significant, but not insurmountable, biosafety concerns which would need to be addressed before lentiviral-based vectors can be used in human subjects, the setting of subjects already infected by HIV-1 may overcome some of the concerns. The recent development of third generation, self-inactivating lentivirus vectors will certainly increase the safety of this vector system significantly (62-64).

7. GENE EXPRESSION

While effective gene transfer is the rate-limiting step for gene therapy using hematopoietic stem cells, stable gene expression is another essential component.

For anti-HIV-1 genes, the requisite expression parameters will vary, based on specific aspects of the anti-HIV-1 gene product (e.g. active as RNA or translated into protein). The level of primary transcripts is a function of the synthesis rate and the stability of the RNA. In general, higher levels of the anti-HIV-1 gene product would be expected to have greater inhibitory effects, although there may be an upper limit above which more product could lead to cellular toxicity. The persistence of expression in mature hematopoietic and lymphoid cells derived from transduced stem cells will be important for achieving an enduring effect. Other factors, such as the intracellular location of the gene product, association with cellular proteins, 2^o and 3^o structure of RNA (e.g. containing ribozymes), etc. may play important roles in determining efficacy.

Initial studies of retroviral-mediated gene transfer into hematopoietic stem cells focused on "housekeeping enzymes" such as ADA and glucocerebrosidase which are ubiquitously expressed in a loosely regulated manner. Expression of these genes under control of constitutive viral promoters, such as the MLV LTR or the CMV or SV40 enhancer/promoters, may result in expression in all transduced cells at variable levels. These exogenous promoters may be subject to “silencing“ in a portion of the transduced cells, in association with DNA methylation and chromatin condensation (65). Our group and others have made modifications in the basic transcriptional control elements of the MLV LTR to produce vectors capable of more sustained, high level expression after transduction of HSC (66-68). The improved expression activity of these laboratory-modified vectors, compared to native murine retroviruses, may help overcome the poor expression in human subjects, which has been seen in a trial in ADA-deficient SCID infants (69). Other investigators have used expression cassettes from polIII promoters (35, 70, 71) with the goals of achieving higher levels of accumulated RNA or directing the RNA product to specific cellular locations to increase the frequency of interactions with target HIV-1 macromolecules.

8. CLINICAL TRIALS

In vitro models of cell transduction and inhibition of HIV-1 can be informative, but fail to test the two key parameters required for successful stem cell gene therapy in HIV-1: 1) efficient gene transduction of true pluripotent human hematopoietic stem cells which can give enduring production of mature progeny cells of lymphoid and myeloid lineages and 2) the anti-viral effects of anti-HIV-1 gene expression in the HIV-1-infected host. Thus, clinical trials must be performed as part of the evaluative process, with a long-term goal being development of clinically beneficial strategies. In general, nearly all clinical studies using MoMuLV retroviral vectors to transduce $CD34^+$ cells (for correction of genetic diseases, marking of reconstituting cells in oncology patients, or for an anti-HIV-1 approach) have shown very low levels of cells in the circulation of subjects (72).

Only a few clinical trials have been performed targeting hematopoietic stem cells from HIV-1 infected subjects. Systemix (a Novartis Corp.) has performed a trial of retroviral-mediated transfer of the RevM10 gene into $CD34^+$ PBSC from HIV-1 infected adults (25). An initial report showed low gene transfer and no detectable cells in the circulation of patients after infusion of transduced cells.

8.1. Transfer of an anti-HIV-1 ribozyme gene into $CD34^+$ PBSC from HIV-1 positive adults

From the long-standing collaboration between our group at Childrens Hospital Los Angeles and the groups of John Rossi and John Zaia at City of Hope National Medical Center, we have progressed through stages of gene discovery, vector design, identification of constructs which consistently inhibit HIV-1 replication, pre-clinical testing, protocol development and review to perform a clinical trial with the anti-HIV-1 hammerhead ribozymes (31, 40, 73). Five subjects have been treated under the protocol. All underwent leukapheresis. The cells of the CD34-enriched fraction were transduced with either the retroviral vector L-TR/TAT (containing the ribozyme) or LN (containing the neo-resistance gene) and given back to their donors by a single intravenous infusion without any adverse effects.

Analysis of peripheral blood samples taken

monthly after cell infusion showed the presence of gene-containing cells in some peripheral blood samples. However, the frequency of gene-containing cells was quite low (less than 1/100,000) and no positive samples were seen later than six months after the cell re-infusions. These observations suggest that there was transduction and engraftment of mature progenitor cells of relatively short-term proliferative capacity, but do not provide evidence for transduction and engraftment of long-lived stem cells.

8.2.Transfer of an RRE decoy gene into $CD34^+$ cells from the bone marrow of HIV-1-positive children

We have performed a pilot study to evaluate the safety, feasibility and efficacy of using retroviral-mediated transduction of an RRE decoy gene into $CD34^+$ cells from the bone marrow of HIV-1 infected children (74). Four subjects 8-17 years of age were recruited and underwent the procedure. For this study, two vectors were used: one encodes an RRE decoy and the bacterial *neo* gene (L-RRE-neo) and one which only contains the *neo* gene (LN). Each patient's cells (0.5-3.2 x 10^6 $CD34^+$ cells/kg), were divided into two portions and one got the RRE decoy, the other got the *neo*-only vector. Cells were transduced by 3 day culture on autologous stroma and IL-3/IL-6/SCF, and returned to the subject by intravenous administration without adverse effects.

Transduction of clonogenic progenitors in the marrow was between 7% and 30%, but the cells with the RRE decoy vector were seen in the peripheral blood only on the first day following cell infusion.

8.3. Transfer of an anti-HIV-1 ribozyme into $CD34^+$ PBSC from adults with HIV-1 and lymphoma

In an ongoing study, investigators at City of Hope are performing autologous transplantation of ribozyme-transduced PBSC in patients with HIV-1 and lymphoma, following myeloablative chemotherapy. Potentially, the cytoablation may allow a greater extent of engraftment from transduced HSC. Initial results in the few months following the procedure have shown higher levels of the ribozyme-containing cells than in the prior studies, with detectable levels of vector-derived transcripts in peripheral blood mononuclear cells (PBMC) and granulocytes. It remains to be determined whether enduring engraftment and production of gene-containing cells will be seen.

8.4. Umbilical cord blood cell (UCBC) trial

It is possible that this procedure could be performed with a higher degree of efficacy using umbilical cord blood cells (UCBC) from HIV-1 infected neonates. While the trials with PBSC and bone marrow have not shown transduction of long-term stem cells, our previous trial using cord blood from ADA-deficient neonates has, with gene-containing T cells, monocytes and granulocytes present in peripheral blood, for more than five years after treatment (69, 75). Thus, cord blood may contain stem cells which are susceptible to transduction by MLV-based vectors and result in long-term production of cells containing the RRE decoy.

In addition to the potentially more effective transduction of umbilical cord blood stem cells compared to those in bone marrow (28), the use of this approach for HIV-1 infected neonates has additional potential benefits. The umbilical cord blood cells should - as transmission is mostly pernatal - contain normal numbers and function of hematopoietic stem cells, which may not be the case in the bone marrow of HIV-1 infected children and adults (29). The development of mature functional T cells from transduced stem cells is likely to require active thymic function. It is known that thymic function is greatest in newborns and declines with age, so that by the second decade of life, thymic function is significantly reduced (76, 77). Additionally, HIV-1 infection itself has been reported to degrade thymic function (78). Therefore, stem cell-directed gene therapy in HIV-1 infected newborns is likely to have the greatest possibility of producing functioning T cells, due to the presence of a young, functional thymus.

One logistic difficulty with using umbilical cord blood to treat HIV-1 infected children is the unpredictable likelihood of active HIV-1 infection developing in an infant born to an HIV-1 infected mothers. Landmark studies have shown that the rate of maternal to infant HIV-1 transmission can be reduced to as low as 10 percent by treatment of the mother with antiretroviral agents (79). It would not be sensible to perform gene therapy in all infants born to HIV-1 infected mothers, if only 1 out of 10 is actually infected. Observation for 1 to 3 months after birth is required before it can be determined whether an infant has become infected by HIV-1. Thus, to perform a clinical trial of gene therapy, the umbilical cord blood needs to be collected from many infants born to HIV-1 infected mothers and cryopreserved during the period until the infection status of the infant can be definitively assessed. When an infant is documented to be infected by HIV-1, their own cord blood specimen can be thawed, transduced and infused.

This schema necessitates establishment of a cord blood bank for collection of cord blood from a large group of HIV-1 exposed infants. We have been involved in an effort to establish such a bank. The Pediatric AIDS Clinical Trial Group (PACTG) has developed a clinical protocol to collect cord blood samples from HIV-1-infected pregnant women enrolled at sites in California. We estimate that 100 infants/year will be born to HIV-1-infected women at these sites. Over the course of 2 years, we should be able to have at least 3-5 samples from infants who are actually infected, assuming a low transmission rate of 2%. Comparing results of gene transfer with umbilical cord blood with the results from the other trials of stem cell gene therapy for HIV-1 already planned which use older recipients and bone marrow or peripheral blood stem cells, it will be possible to make some comparisons to determine whether gene therapy for HIV-1 infection is more effective in infants using umbilical cord blood.

9. PERSPECTIVE

To date, no study involving gene transfer into human hematopoietic stem cells, including those in HIV-1

positive patients, has achieved a therapeutic effect. Delivery systems need to be developed that allow more efficient gene transfer and long-lasting transgene expression.

With the construction of lentiviral vectors, it seems possible to use HIV-1's ability to infect quiescent cells to achieve these goals. Additionally, HIV vectors have been shown to display intrinsic anti-HIV activity, in part thought to be due to TAR and RRE decoy effects (80). However, there are significant safety concerns that need to be addressed before a clinical application is conceivable. There is the potential risk of the occurrence of replication competent retrovirus (RCR) during vector production and of lentiviral vector mobilization by HIV-1 once expressed in the same cell. The development of third generation, self-inactivating vectors (62-64) significantly reduces these risks. The use of nonprimate lentiviral vectors, such as the feline immunodeficiency virus (FIV) (81) or a vector combining human and simian immunodeficiency virus elements (82) might also prove useful.

New approaches need to be pursued regarding the anti-HIV genes used. Designing lentiviral vectors containing anti-HIV genes poses the problem of not only targeting the patient's HIV but also interfering with the vector production. Since most vectors use VSV-G pseudotype, the HIV envelope might be a possible target. *Rev*-independent vectors, such as non-HIV-1 lentiviruses or HIV-viruses containing constitutive transport elements (CTE) to replace *rev*, may be used to target HIV-*rev*.

Finally, more clinical trials are needed to evaluate the significance of the *in vitro* findings in the actual clinical situation.

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Send correspondence to: Donald B. Kohn, M.D., Mailstop # 62, Childrens Hospital Los Angeles, 4650 Sunset Blvd., Los Angeles, CA 90027, Tel: 323-669-4617, Fax:323-667-1021, E-mail: dkohn@chla.usc.edu and bce@hsc.usc.edu

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