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Gene- and Immunotherapy for Hematological Diseases

Editor
Dagmar Dilloo, Düsseldorf

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Guest Editor
Dagmar Dilloo, Düsseldorf

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Acta Hæmatologica

Contents

59 **Editorial**

60 **Gene Therapy for Inborn and Acquired Immune Deficiency Disorders**
Engel, B.C.; Kohn, D.B. (Los Angeles, Calif.)

71 **Gene Transfer Strategies for Correction of Lysosomal Storage Disorders**
d'Azzo, A. (Memphis, Tenn.)

86 **Gene Therapy for Chronic Granulomatous Disease**
Goebel, W.S.; Dinauer, M.C. (Indianapolis, Ind.)

93 **Hematoprotection by Transfer of Drug-Resistance Genes**
Flasshove, M.; Moritz, T.; Bardenheuer, W.; Seeber, S. (Essen)

107 **Gene Therapy Targeting Hematopoietic Cells: Better Not Leave It to Chance**
Baum, C. (Hannover/Cincinnati, Ohio); von Kalle, C. (Cincinnati, Ohio)

110 **Adoptive Immunotherapy in Chimeras with Donor Lymphocytes**
Kolb, H.-J.; Schmid, C.; Chen, X.; Woiciechowski, A.; Roskrow, M.; Weber, M.; Guenther, W.; Ledderose, G.; Schleuning, M. (Munich)

121 **Cellular Engineering of HSV-tk Transduced, Expanded T Lymphocytes for Graft-versus-Host Disease Management**
Burger, S.R. (Chapel Hill, N.C.); Kadidlo, D.M.; Basso, L.; Bostrom, N.; Orchard, P.J. (Minneapolis, Minn.)

132 **Alternative Concepts of Suicide Gene Therapy for Graft-versus-Host Disease after Adoptive Immunotherapy**
Kramm, C.M. (Düsseldorf)

139 **Adoptive T-Cell Therapy for EBV-Associated Post-Transplant Lymphoproliferative Disease**
Bollard, C.M.; Savoldo, B.; Rooney, C.M.; Heslop, H.E. (Houston, Tex.)

149 **Adoptive T-Cell Therapy for Epstein-Barr Virus-Positive Hodgkin's Disease**
Huls, M.H.; Rooney, C.M.; Heslop, H.E. (Houston Tex.)

154 **Chimeric T-Cell Receptors for the Targeting of Cancer Cells**
Rössig, C. (Münster); Brenner, M.K. (Houston, Tex.)

160 **Leukemia Vaccines**
Glouchkova, L.; Ackermann, B.; Dilloo, D. (Düsseldorf)

171 **Author Index, Vol. 110, No. 2–3, 2003**
172 **Subject Index, Vol. 110, No. 2–3, 2003**

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Editorial

Over the last years, gene and immunotherapeutic strategies for the treatment of hematological diseases have been successfully transferred from the laboratory to the clinic. The aim of this issue of *Acta Haematologica* is to provide an overview on gene and immunotherapy for non-malignant and malignant hematological diseases as alternative or complimentary treatment to standard therapy. Experts in the field have contributed to cover the broad scope of currently available strategies ranging from corrective gene transfer for monogenetic diseases to adoptive transfer of T lymphocytes and vaccine development in immunotherapy of leukemia and lymphoma.

In gene therapy for monogenetic disorders of the hematopoietic system, the initial limitations of low gene transfer efficiency have been successfully addressed. Now that therapeutically effective levels of gene transfer can be achieved, the use of autologous, genetically modified hematopoietic cells has opened new therapeutic avenues for affected patients without the risks associated with allogeneic stem cell transplantation. Model diseases affecting the lymphopoietic system, such as immunodeficiencies, or the myelopoietic system, such as chronic granulomatous disease or metabolic storage disorders, have been chosen to illustrate the therapeutic potential of stable gene transfer into hematopoietic cells for correction of genetic disorders. In this context chemoresistant genes for selection of successfully transduced target cells may prove beneficial, a strategy explored in another review. In the clinical application of corrective gene transfer, the initial focus was on vector safety. To date, with a considerable number of patients treated and the development of lymphoproliferative disorders in 2 children in a French trial for gene therapy of severe combined immunodeficiency, issues such as insertional mutagenesis as well as disease or target cell-specific susceptibility to gene transfer toxicity have moved into the foreground. Included therefore is a short commentary addressing the current safety issues.

In immunotherapy of leukemia and lymphoma, both genetically modified and non-modified effector cells are employed. Prominent examples of clinically successful adoptive T-cell therapy are Epstein-Barr virus-specific cytotoxic T cells for the treatment of lymphoproliferative and Hodgkin's disease and donor lymphocytes for relapsed chronic myeloid leukemia. Genetic modification of T cells is pursued with a number of different goals such as gene marking for long-term tracking of administered cytotoxic T lymphocytes or transgenic expression of suicide genes to provide a rescue mechanism in case of graft-versus-host disease following donor lymphocyte infusion. Also, the potential of retargeting T cells toward leukemia/lymphoma-specific antigens via gene transfer of hybrid T-cell receptors is reviewed as an alternative strategy to classical cytotoxic T-cell generation. In highly proliferative diseases, such as high-risk acute leukemia, adoptive T-cell therapy is considerably less effective so that for these patients relapse prevention in the setting of minimal residual disease is paramount. Here, vaccine strategies with the aim to stimulate a leukemia-specific immune response in vivo may prove advantageous and the various approaches to leukemia vaccine generation are discussed.

While the transfer of gene and immunotherapy to the clinic has provided promising initial results for a number of hematological diseases, researchers have now been confronted with new challenges in terms of toxicity. Yet, based on careful consideration of potential toxicity and detailed analysis of adverse events, it is the continuous pursuit of these novel treatment strategies in the framework of clinical studies that will ultimately enable progress in this field.

Dagmar Dilloo

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Gene Therapy for Inborn and Acquired Immune Deficiency Disorders

Barbara C. Engel[a] Donald B. Kohn[a,b]

[a]Division of Research Immunology/Bone Marrow Transplantation, Children's Hospital Los Angeles, and
[b]Department of Pediatrics, University of Southern California Keck School of Medicine, Los Angeles, Calif., USA

Key Words
Gene therapy · Immune deficiency · Hematopoietic stem cells

Abstract
Gene therapy has been under development as a way to correct inborn errors for over 20 years. Immune deficiencies are favorable candidates for gene therapy because of the potential selective advantage of genetically corrected cells in these conditions. Gene therapy for immune deficiencies has been the only application to show incontrovertible benefit in clinical trials to date. Despite the success in treating the underlying disease, there have been two cases of insertional oncogenesis reported in one of these early phase trials. Gene therapy approaches and clinical trials for several inborn as well as acquired immune deficiencies will be reviewed.

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Gene Therapy for Inborn Immune Deficiency Disorders

Gene therapy has been under development as a way to correct inborn errors for over 20 years [1]. In the early 1980s, as gene therapy became technically feasible, hemoglobinopathies were the first diseases to be considered because the human β-globin gene was the first human gene to be cloned. However, by the mid 1980s it became clear that gene transfer into primary human hematopoietic stem cells (HSC) was inefficient with the available techniques. Consideration turned to the congenital immune deficiencies as more favorable candidates because of the potential selective advantage of genetically corrected cells in these conditions. Gene therapy for immune deficiencies continues to be a major focus of research and, as will be discussed, has been the only one to show a clear-cut significant benefit in clinical trials to the present time.

The congenital condition known as 'severe combined immune deficiency' (SCID) was the first human disease to be successfully cured by allogeneic bone marrow transplant in 1968 [2]. From the accumulated experience with transplants for SCID, it became clear that there is a potent selective advantage for genetically normal donor T-lymphoid progenitors in SCID patients [3]. Intravenous administration of only a modest number of normal bone

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Barbara C. Engel
Mailstop # 62, Children's Hospital Los Angeles
4650 Sunset Blvd., Los Angeles, CA 90027 (USA)
Tel. +1 323 669 2237, Fax +1 323 671 3651
E-Mail bengel@chla.usc.edu

marrow cells without any prior cytoreductive conditioning routinely leads to complete reconstitution of the immunologic system with donor-derived T lymphocytes. These findings implied that there was a selective advantage for the normal donor T cells, perhaps because of the available open niche lacking recipient's endogenous T cells. From these observations of selective advantage in SCID, it was proposed that a similar selective advantage for genetically corrected autologous T-lymphoid progenitors may be expected in gene therapy for SCID and that this selective advantage could amplify the effects from gene transfer into only a small number of HSC [3].

Adenosine Deaminase-Deficient SCID

In the mid 1980s, the only known and cloned gene which was causative for a form of human SCID was that encoding adenosine deaminase (ADA). Retroviral vectors based on the Moloney murine leukemia virus were developed to carry the normal human ADA cDNA, and studies showed that transfer of the normal ADA cDNA into ADA-deficient T cells corrected the physiologic defect in vitro [4].

Although initial considerations had been towards targeting pluripotent HSC with the corrective gene to provide a renewing source of T-lymphoid progenitors, it became apparent that stem cells were a difficult target. At that time, there was no way to achieve a significant enrichment of HSC, there was no cloned recombinant hematopoietic growth factor which could be used to stimulate HSC to proliferate and take up retroviral vectors and there were insufficient assays to measure stem cell activity in preclinical studies. Therefore, a group of investigators at the National Institutes of Health, lead by French Anderson and Michael Blaese, turned to the mature T cells as the potential target for gene transfer [5].

An effective form of enzyme replacement therapy had been developed for ADA-deficient SCID consisting of bovine ADA which is purified and conjugated to polyethylene glycol (PEG-ADA) [6]. Administration of PEG-ADA by biweekly intramuscular injections can lead to significant restoration of immunity in the majority of ADA-deficient SCID patients, with significant increases in the numbers of peripheral blood T lymphocytes.

The presence of circulating T cells in ADA-deficient SCID subjects receiving PEG-ADA allowed their T cells to be collected by phlebotomy or apheresis, targeted in vitro with a retroviral vector and then reinfused into the patients. This unique opportunity to access T cells in SCID patients who are otherwise severely lymphopenic formed the basis for the first clinical trial of human gene therapy which began in 1990 [5]. Two patients were treated under this protocol. They underwent multiple cycles of peripheral blood T-cell collection, ex vivo transduction, and reinfusion.

Importantly, there were no serious adverse events from using this approach. One of the most feared complications of the use of retroviral vectors was that the presence of replication-competent retrovirus (helper virus) could lead to a productive infection that may be especially devastating in patients with immune deficiency. In fact, no replication-competent retrovirus was seen in these patients or in the over 1,000 patients who have undergone retroviral vector-mediated gene transfer to the present time. In 1 of the patients, increased levels of ADA enzymatic activity (up to 25% of normal) were expressed in circulating peripheral blood T cells. These ADA gene-containing and expressing peripheral blood T lymphocytes persist through the present time, more than 10 years since the trial began. This long-lasting persistence of T cells that were isolated, manipulated and reinfused is an important observation and supports the potential for this approach. However, the patients have been maintained on PEG-ADA therapy to the present time, due to concerns that the use of mature T lymphocytes may result in a limited immunologic repertoire of gene-corrected T cells with minimal ability to generate new T-cell specificity. Subsequently, a number of ADA-deficient SCID subjects in Italy and Japan were treated under a similar protocol targeting T cells, with similar results achieved [7, 8].

The first trial targeting HSC with the ADA gene was initiated in 1992 by Bordignon et al. [7] in Italy. This study was cleverly designed to attempt to directly compare the efficacy of gene transfer into stem cells versus T lymphocytes. Two retroviral vectors carrying the ADA gene were produced which were nearly identical but could be distinguished on the basis of restriction site polymorphisms. One vector was used to transduce peripheral blood T cells in a protocol similar to that used at the National Institutes of Health (NIH) while the second vector was used to transduce HSC and both cell populations were then also reinfused. Initially, most circulating T cells that contained a vector were from the transduced T cells, but, over time, there was more contribution to peripheral blood T cells from the transduced bone marrow. While a follow-up of these patients has not been published, they do not appear to have had clinical benefit from the procedure. Shortly after the study began in Italy, 3 patients from France and England were treated with retroviral-mediated transfer of the ADA gene into bone marrow cells [9]. The level of gene transfer was quite low and there

was minimal evidence for production of gene-containing T cells in the patients.

In 1993, our group at the Children's Hospital Los Angeles performed a trial of retroviral-mediated ADA gene transfer into CD34+ cells derived from the umbilical cord blood of 3 ADA-deficient newborns [10]. The subjects have had a low but persistent number of gene-containing peripheral blood stem cells now for over 8 years. An attempt was made to wean the patients off PEG-ADA, and during this time, the frequency of gene-containing T lymphocytes increased significantly. This finding implied that the PEG-ADA therapy blunted the selective survival advantage of the gene-corrected cells, which only became apparent as the PEG-ADA enzyme was weaned. An attempt to completely eliminate PEG-ADA administration in 1 of the subjects was halted after he developed early signs of immune deficiency with oral thrush [11]. Subsequent studies show that there was minimal expression of the ADA gene from the vector in circulating peripheral blood T cells, but expression could be induced to a high level by in vitro culture and activation with T-cell mitogens [11]. To the present time, no patient with ADA-deficient SCID has had a significant clinical benefit produced from gene transfer into either T cells or peripheral blood stem cells, although some degree of gene transfer and expression has been documented.

We are currently performing a second trial for ADA-deficient SCID in collaboration with Fabio Candotti, MD, at the NIH. We are exploring the efficacy of new cytokines (e.g. flt-3 ligand, thrombopoietin) which may be better able to stimulate gene transfer to stem cells and new vectors which may be better expressed in quiescent T cells. A recent report by Aiuti et al. [12] on 2 subjects undergoing retroviral-mediated transfer of the ADA gene into CD34+ cells with prior administration of busulfan for cytoreduction and no PEG-ADA therapy demonstrates immune reconstitution.

X-Linked SCID

The most common single genetic etiology of SCID is the X-linked form which is due to the absence or deficiency of the γC cytokine receptor responsible in part for signaling from IL-2, IL-4, IL-7, IL-9, IL-15 [13]. The γC gene was identified and cloned in 1992 [14]. Hacaein Bey et al. [15] performed pre-clinical studies showing that retroviral-mediated gene transfer of a normal γC gene into T cells and HSC from patients with X-linked SCID restored cytokine signaling and restored lymphopoiesis. These pre-clinical studies led to a clinical trial in which a total of 11 infants or children with X-linked SCID were treated by gene transfer into their autologous HSC [16–18]. In 10 of the patients, significant production of peripheral blood T, B and NK cells containing the normal gene was seen with restoration of essentially normal immunologic function. The 1 subject who did not realize a benefit had massive hepatosplenomegaly due to a preexisting infection and failed to show engraftment of the transduced cells. The findings in these X-SCID patients exemplify the postulated selective advantage in SCID with immune reconstitution presumably from a small number of gene-corrected HSC. X-SCID may have a stronger selective advantage for gene-corrected cells than ADA-deficient SCID and the absence of an enzyme replacement therapy for X-SCID obviates any blunting of selective advantage that is seen with PEG-ADA for ADA-deficient SCID. All of the 10 patients are alive and have been able to lead a normal life for up to 3 years for the first group of patients treated. The long-term duration of the clinical benefit remains to be seen but the initial success in treating their X-linked SCID is clear and incontrovertible. However, about 30 months after treatment, 2 of the patients in the first group of 5 developed a T-cell leukemia (see online press releases by the American Society of Gene Therapy and the European Society of Gene Therapy). In both cases, this seems related to insertion of the retroviral vector in or near a gene called LMO-2. This gene is known to cause T-cell leukemia if activated inappropriately, e.g. as a result of a chromosomal translocation [19, 20]. Both patients have been treated with chemotherapy and so far seem to have responded to the treatment.

Pre-clinical studies of the gene therapy approach used in this X-SCID study had shown no evidence of cancer, leukemia or otherwise, nor has such an event ever been reported in any of the other clinical trials using retroviral vectors targeting HSC. The key scientific issue to resolve is why this has only occurred in this X-SCID study. In vitro and animal studies will have to address the questions whether this is disease/gene-specific (γC), a problem in the gene transfer method or a combination of both.

The genes responsible for many other genetic forms of SCID have also been cloned and studies in animal models have shown that other forms of SCID are equably amenable to therapy. Thus, it is possible that within the next few years, gene therapy will be applied successfully to Jak-3-deficient SCID, Rag1/Rag2-deficient SCID, as well as other rare forms. Development of gene therapy for these diseases may be limited by their extremely rare nature, making it unattractive to invest the time and money needed to develop gene therapy for a condition where it may be difficult even to recruit patients. Depending on

the results regarding the mechanism of the leukemia-development in the X-SCID study, there may be impetus to do so.

Chronic Granulomatous Disease

Another immune deficiency disease to be approached by gene therapy is chronic granulomatous disease (CGD). CGD is characterized by repeated pyogenic infections due to the absence of neutrophil oxidase activity involved in intracellular killing of bacteria and other pathogens [21]. There are both autosomal as well as X-linked forms of CGD, indicating the existence of multiple causative gene loci. Gene transduction of HSC in murine models of CGD have shown effective restoration of neutrophil oxidase activity with protection from pathogenic bacteria [22, 23]. It is likely that it would not be necessary to correct a large number of HSC, as a clinical benefit could be realized from a relatively modest percentage of functionally active neutrophils. Clinical trials have been performed in CGD by Malech et al. [24] at the NIH. It was possible to demonstrate the production of functionally normal neutrophils with restored oxidase activity but these were present at low numbers and only for short times after infusion of gene-modified cells. Gene therapy approaches for CGD are covered separately by Goebel and Dinauer [pp 86–92] and will not be further addressed here.

Additional Diseases

A variety of other congenital immune deficiencies may be excellent candidates for gene therapy directed at HSC. Wiskott-Aldrich syndrome (WAS) is a complex hematologic disorder in which there is dysfunction of T cells, B cells, macrophages, as well as platelets. The gene responsible for WAS, WASP, has been cloned and appears to act at the interface of cellular cytoskeleton and signal transduction pathways [25]. Studies in WAS patient-derived cells as well as in a WASP gene knock-out mouse model have shown that transfer of the WASP cDNA can correct many of the manifestations of the cellular defects [26, 27]. It is likely that there will be a strong selective survival advantage for WASP gene-corrected cells, in that women who are heterozygous for the WASP defect show non-random X inactivation patterns, consistent with strong selective outgrowth of lymphocytes in which the normal WASP allele did not undergo lyonization.

Another congenital immune deficiency which may be a good candidate for gene therapy targeted to HSC is X-linked agammaglobulinemia (XLA). XLA results from a defect in a protein called Bruton's tyrosine kinase or *btk*, that is involved in B-lymphocyte signal transduction and maturation [28]. In the absence of *btk* function, B-lymphocyte development is arrested at the early pre-T-cell phase. Restoration of *btk* activity by gene transfer should allow B-cell development to proceed. However, there is potential that constitutive expression of *btk*, which is normally expressed only transiently in B-cell development, may lead to cellular dysfunction or malignant overgrowth. This has not been seen in murine studies performed to date, although the level of expression has been relatively low. *btk* expression should confer selective proliferation on the gene corrected B-lymphoid progenitors, again potentially allowing a clinically beneficial result to occur from gene transduction of a modest number of stem cells.

Other even rarer genetic immune deficiencies such as the X-linked lymphoproliferative syndrome, leukocyte adhesion deficiency, CD40 ligand deficiency (X-linked hyper-IgM syndrome) and other disorders of leukocyte production or function are also likely to be candidates for gene therapy. Treatment of the specific disorder requires identification and isolation of the normal responsible gene, determination of the extent of gene transfer and expression necessary for correction of the condition, testing in appropriate animal or cell culture pre-clinical models, and then the performance of clinical trials to test safety and efficacy. It may be difficult to identify resources necessary to perform all of these pre-clinical and clinical studies for these relatively rare disorders. However, besides the direct benefits of these studies to the patients who suffer from these diseases, it is expected that these studies will lead to effective gene therapy for more common disorders, such as sickle cell disease, thalassemia, and lysosomal storage disorders.

Gene Therapy for Acquired Immune Deficiency Syndrome

The human immunodeficiency virus (HIV-1) infects human CD4+ T lymphocytes and monocytic cells. Although improvements in anti-retroviral medications have produced great benefits to many people infected with HIV-1 [29–32], these drugs are costly and may have severe side effects. Treatment must be lifelong for long-term protection of patient's CD4+ T lymphocytes from viral destruction to prevent the occurrence of severe and life-threatening opportunistic infections and neoplasms. Therefore, development of alternative or complementary treatments for HIV-1 is needed. With the emergence of

the field of gene therapy, applications to treatment of infectious diseases, such as HIV-1 infection, are being explored.

Target Cells

The optimal target cell for gene therapy of HIV-1 infection has not yet been identified. Initial clinical trials used peripheral blood CD4+ T lymphocytes that are relatively easy to obtain and, unlike HSC, relatively easy to transduce [33]. Because mature T cells have a limited lifespan, repeated infusions of gene-modified cells would be required to maintain protection, posing logistical and financial problems.

In addition to CD4+ T lymphocytes, other cells such as T-cell precursors in the thymus and lymph nodes, as well as macrophages, dendritic cells and microglia in the brain are also infected during the course of AIDS. All of these cells are derived from HSC in the bone marrow [34, 35]. Gene transfer into HSC (from bone marrow, peripheral blood or cord blood) would theoretically create a pool of cells that could give rise to all hematopoietic lineages and confer lifelong antiviral protection to the descendant cells [36].

If essentially all of a patient's HSC and the resultant T lymphocytes and monocytic cells could be rendered unable to support HIV-1 replication, it is likely that viral loads would decrease. Theoretically, inhibition of HIV-1 replication in 99.9% of the susceptible cells would be necessary to produce a 3-log reduction in virus burden, an effect often seen with highly effective anti-retroviral therapy. However, with the limited capabilities to effectively transduce high percentages of human HSC, it is currently not possible to protect the majority of susceptible cells. Another mechanism to achieve efficacy would be to engineer cells that are incapable of supporting active HIV-1 replication and are protected from virus-induced cytopathicity, thus having a selective survival advantage compared to non-protected cells. In this case, a modest number of protected cells may comprise an increased percentage of all T lymphocytes, leading to some preservation of immune function. This selective survival of gene-protected T cells has been observed in vivo in a study by Woffendin et al. [33] who demonstrated a selective survival advantage for T lymphocytes expressing a dominant-negative *rev* gene (revM10).

Anti-HIV-1 Genes

As opposed to the conventional immunization techniques in which an entire organism is protected against invasion by a microbial agent, the concept of 'intracellular immunization' involves genetic modification of cells that would be potential targets for viral infection [37]. Genetic elements that inhibit viral replication, so-called resistance genes, are stably transferred into a patient's cells. Ideally, these resistance genes should be effective, nontoxic and not bypassed by HIV-1 sequence variation.

A number of synthetic genes have been developed that show suppression of HIV-1 replication in model systems. Generally there are three possibilities of intervention with the HIV-1 viral life cycle: preventing the virus from getting into the cells, interfering with its replication once it has infected the cell, and, finally, destroying infected cells. Both protein-based and RNA-based anti-HIV-1 gene products have been developed and are currently being studied in various clinical trials.

Protein-Based Strategies

Inhibition of Viral Entry. In addition to the CD4 receptor, HIV-1 requires chemokine receptors as co-receptors for entry into the cell [38, 39]. It has been shown that the chemokine receptor CCR5 is used by macrophage-tropic virus strains, whereas T-cell-tropic viruses (that emerge later in disease progression) use the CXCR4 receptor. Several approaches have been under investigation to prevent the virus from getting into the target cell including attempts to downregulate co-receptor expression using intracellular single-chain variable fragments, ribozymes, or intrakines [40–45]. Another recently described strategy involves a membrane-anchored peptide to block the interaction of gp41 with the cell membrane [46]. Systemic administration of the peptide T20 has previously been shown to effectively inhibit HIV-1 entry. Although initial results seem promising, more information has to be gained about these approaches to anti-HIV-1 therapy. It is conceivable that HIV-1 mutants might be selected that resist the blockade to the CCR5 co-receptor encouraging the development of a more pathogenic virus that uses CXCR4 [47].

Transdominant Mutant Proteins. Transdominant negative mutant proteins are mutant forms of essential HIV-1 proteins which can interfere with the function of the wild-type HIV-1 protein to suppress viral replication. The concept is that a partially homologous mutant protein can still interact with wild-type polypeptide chains but is otherwise defective. In this scenario, only a few mutant monomers may be sufficient to recruit multiple wild-type monomers into nonfunctional multimers. Alternatively, the mutant proteins can compete with the wild-type protein for cofactors, substrates or targets available only in limited amounts within the cell. This

approach has been described for both regulatory (Tat, Rev) and structural (Gag, Env) HIV-1 proteins and cofactors [48–54].

Inducible Intracellular Toxic Proteins. The principle of this method is to kill HIV-infected cells. Sequences encoding toxic proteins (like herpes simplex thymidine kinase or attenuated diphtheria toxin) are introduced into a patient's cells under the control of the HIV-long terminal repeat (LTR) promoter, which is activated by the TAT protein expressed by HIV-1. Upon infection with HIV, the production of the HIV-1 TAT protein triggers the production of the toxin causing 'suicide' of the infected cell [55–59].

Single-Chain Antibodies. Single-chain variable fragments (SFv) of an antibody are fusion proteins of the minimal domains from the heavy and light chains that retain the antigen-binding properties of the parental antibody. When expressed intracellularly, these SFv can bind the viral protein against which they are directed (e.g. Rev, gp120) and trap them in an inappropriate cellular compartment [60–63].

Chimeric Receptors – CD4ζ. In these chimeric immune receptors, the zeta (ζ) subunit of the CD3 T-cell receptor (cytoplasmic domain, necessary for signal transduction) is fused to the extracellular domains of human CD4. Upon binding HIV-1 env (gp120), transduced cells would act as HIV-specific T cells, in an major histocompatibility complex (MHC)-unrestricted manner [64, 65].

There are several potential problems that may be encountered using protein-based strategies. The obvious concern in using inducible intracellular toxic proteins is that uncontrolled activation of the toxic gene may be harmful to the patient. Some studies suggest that intracellular SFv binding to differing epitopes of a specific protein may have different influences on that protein's function that could potentially even enhance HIV-1 production [66]. Generally, the introduction of a foreign protein intended to inhibit HIV-1 might lead to intracellular processing into antigenic peptides which are then presented by MHC class-I molecules, resulting in a cytotoxic T lymphocyte response to the modified cell. Thus, an immune response, which is desirable in various gene therapy approaches to cancer, would in this case lead to the destruction of the resistant cell. In this respect, resistance genes expressing RNA (antisense, ribozymes, decoys) rather than foreign proteins may be preferable as they are expected to be less immunogenic.

RNA-Based Strategies

Antisense RNA. This strategy utilizes expressed RNA sequences that are complementary to viral RNA sequences. By binding to its 'sense' RNA, the antisense RNA can prevent gene expression by interfering with RNA processing or by inhibiting initiation of translation. This approach has the advantage of specificity. With varying degrees of success, antisense RNA has been directed against tat, rev, gag, env, TAR, RRE, the primer binding site, the polypurine tract and parts of the HIV-1 LTR [67–73].

Ribozymes. Ribozymes are small antisense RNA molecules capable of cleaving specific RNA sequences in a catalytic reaction. They have the advantage of being sequence specific. Additionally, as opposed to antisense RNA which needs to be present in excess of its target sequence, one ribozyme can cleave several target RNA molecules by sequential binding, due to the catalytic nature of the reaction. Sequences which have been targeted include tat, rev, gag, integrase, RT and the HIV-1 LTR [74–78]. Apart from the difficulties of achieving specific delivery to the cells, a problem with both technologies – antisense RNA and ribozymes – is the development of escape mutants by changes of one to a few base pairs in the target HIV-1 sequence and the formation of secondary or tertiary structures or protein binding of the target RNA sequence which might then become inaccessible.

RNA Decoys. RNA decoys exploit regulatory processes that are unique to the replication of HIV. Virus replication requires binding of the two key regulatory proteins Tat and Rev to TAR and RRE RNA sequences. RNA decoys compete with the viral RNA for binding of these proteins. By overexpression of these RNA sequences which bind the HIV-1 protein, the proteins are trapped by the artificial targets and cannot bind their natural target sequence on the HIV-1 transcripts [79–82].

One major concern about this strategy is that normal cellular factors can also associate with the decoys and be sequestered upon overexpression of these RNA sequences. To that end, shorter RNA decoys have been constructed that retain the minimal Rev-binding domain and are less likely to bind to other cellular factors but are able to inhibit HIV-1 replication [83, 84].

Gene Delivery

Depending on the target cell, different methods of gene delivery need to be used. T cells can be readily transduced with retroviral vectors upon activation with, for example, monoclonal antibodies to CD28 and anti-CD3 [64]. In contrast, HSC are more difficult to efficiently and stably

transduce. Retroviral vectors require cell division for their integration. As the pluripotent HSC are largely quiescent, transduction efficiency into these cells has been very low [85]. Clinical trials employing retroviral vectors have revealed a transduction efficiency of 0.1–1% into reconstituting stem cells [85].

Approaches to pseudotype the standard MoMuLV-based vectors with the envelope protein of the gibbon ape leukemia virus [86] resulted in moderately higher levels of gene transfer to human cells. 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 [87–91]. Combinations of these techniques have resulted in modest, yet significant, increases in gene marking in primate stem cell transplant models (e.g. 1–10%) [92, 93]. However, even higher levels of gene transduction of stem cells are probably needed for effective treatment of many disorders.

Vector systems based on lentiviruses, such as HIV-1, have been shown to be able to transduce quiescent cells such as neurons, hepatocytes, and others [94]. Pseudotyping HIV-1-based vectors with VSV-G protein has been shown to produce virions with sufficient physical stability to allow concentration to high titers by ultracentrifugation [94, 95]. Studies examining the ability of lentiviral vectors to transduce primitive human hematopoietic progenitor cells have shown great promise for transduction of cells which engraft immune-deficient NOD-SCID mice [96]. They also transduce quiescent CD34+CD38– progenitors which will grow in extended long-term initiating cell culture [97] and can do so following a single exposure of cells to vectors on the day of isolation. Despite all these encouraging results, it is not yet known whether they are really superior to retroviral vectors in transduction efficiency of human HSC that lead to sustained hematopoietic reconstitution in vivo.

Clinical Trials

While in vitro models of cell transduction and inhibition of HIV-1 can be informative, they fail to test the two key parameters required for successful stem cell gene therapy in HIV-1: (1) efficient gene transfer into true pluripotent human HSC which can give lasting production of mature progeny cells of lymphoid and myeloid lineages, and (2) the effects of anti-HIV-1 gene expression on antiviral effects in the HIV-1-infected host. Thus, clinical trials must be performed as part of the evaluative process, with a long-term goal being the development of clinically beneficial strategies. In general, nearly all clinical studies using Moloney murine leukemia virus-based retroviral vectors to transduce CD34+ cells have shown very low levels of gene-containing cells in the circulation of subjects [98].

Only a few clinical trials have been performed targeting HSC from HIV-1-infected subjects. Systemix Inc. has performed a trial of retroviral-mediated transfer of the RevM10 gene into CD34+ peripheral blood stem cells (PBSC) from HIV-1-infected adults. An initial report showed low gene transfer and no detectable cells in the circulation of patients after infusion of transduced cells [99].

In a collaboration between our group at the Children's Hospital Los Angeles and the groups of Zaia et al. [100] at City of Hope National Medical Center, we have performed two clinical trials, transducing PBSC with anti-HIV-1 hammerhead ribozymes. In the first trial [100], 5 asymptomatic HIV-1(+) subjects were treated under the protocol. CD34-enriched peripheral blood hematopoietic cells were transduced with either the retroviral vector L-TR/TAT (containing two hammerhead ribozymes) or LN (containing the neo-resistance gene) and given back to their donors by a single intravenous infusion without any adverse effects. The frequency of gene-containing cells in follow-up studies of peripheral blood was quite low (less than 1/100,000) and no positive samples were seen later than 6 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 no transduction and engraftment of long-lived stem cells.

In a second trial [100], the investigators at City of Hope have performed autologous transplantation of ribozyme-transduced PBSC in patients with HIV-1 and lymphoma, following myeloablative anti-lymphoma chemotherapy. Potentially, cytoablation may allow greater engraftment of transduced HSC. Higher levels of the ribozyme-containing cells than in the prior studies were observed, with detectable levels of vector-derived transcripts in peripheral blood mononuclear cells and granulocytes. However, as seen in the first trial, there was no detectable gene marking after 6–12 months.

We have also 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 [101]. Four subjects, 8–17 years of age, were recruited and underwent the procedure. For this study, two vectors were used: one encoding an RRE decoy and the bacterial *neo* gene (L-RRE-neo), and one encoding only the *neo* gene

(LN). Each patient's cells were divided into two portions, one portion received the RRE decoy gene, the other portion the *neo*-only vector. Cells were transduced by 3-day culture in IL-3/IL-6/SCF on autologous stroma, 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.

We have started a new phase-I clinical trial of gene transfer of anti-HIV-1 gene into bone marrow CD34+ cells from HIV-1-infected children. Retroviral vectors carrying the huM10 'humanized' dominant-negative *rev* gene (or a control, nonexpressed gene) are being used. The '2nd generation' conditions for transduction of CD34+ cells (flt-3 ligand/TPO/SCF on recombinant fibronectin) described above are being used. Pediatric subjects of 3–13 years of age with relatively early HIV-1 infection are the study population. This age group was chosen because young children would have the greatest probability of developing new T lymphocytes from transduced HSC due to their greater thymic capacity. Thus, they could derive a clinical benefit if T lymphocytes expressing huM10 have prolonged survival, whereas this potential benefit for children could be missed if only older subjects (e.g. adolescent or adult), with poorer thymic function, were studied. The trial is approved for a total of 12 subjects, 6 with plasma HIV-1 levels <1,000 copies/ml and 6 >1,000/ml. Two children have been enrolled (November 2000 and February 2001) and undergone the procedure to date. Bone marrow was harvested, CD34+ cells isolated and transduced and the cells reinfused without adverse events. Follow-up studies are in progress.

As of today, no study involving gene transfer into cells of HIV-1-positive patients has achieved a significant therapeutic effect. New improved tools need to be developed to allow better gene transfer as well as long-lasting transgene expression. With the development of lentiviral vectors, it might be possible to use the ability of HIV-1 to infect nondividing cells to achieve these goals, provided the safety concerns in this particular setting can be resolved. There is a potential for the mobilization of lentiviral vectors in patient's cells that are co-infected with wild-type-HIV-1, which could increase the risks of recombination. While the development of third generation SIN vectors [102–104] reduces these risks [105], careful studies will need to be done before this vector system can be applied in this disease setting.

For all retroviral studies targeting HSC, careful consideration will have to be made of the benefits versus risk ratio for each disease under study.

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Gene Transfer Strategies for Correction of Lysosomal Storage Disorders

Alessandra d'Azzo

Department of Genetics and Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, Tenn., USA

Key Words
Lysosomal enzymes · Lysosomal storage diseases · Gene therapy · Viral vectors · Animal models

Abstract
Lysosomal storage diseases (LSDs) represent a large group of monogenic disorders of metabolism, which affect approximately 1 in 5,000 live births. LSDs result from a single or multiple deficiency of specific lysosomal hydrolases, the enzymes responsible for the luminal catabolization of macromolecular substrates. The consequent accumulation of undigested metabolites in lysosomes leads to polysystemic dysfunction, including progressive neurologic deterioration, mental retardation, visceromegaly, blindness, and early death. In general, the residual amount of functional enzyme in lysosomes determines the severity and age at onset of the clinical symptoms, implying that even modest increases in enzyme activity might affect a cure. A key feature on which therapy for LSDs is based is the ability of soluble enzyme precursors to be secreted by one cell type and reinternalize by neighboring cells via receptor-mediated endocytosis and routed to lysosomes, where they function normally. In principle, somatic gene therapy could be the preferred treatment for LSDs if the patient's own cells could be genetically modified in vitro or in vivo to constitutively express high levels of the correcting enzyme and become the source of the enzyme in the patient. Both ex vivo and in vivo gene transfer methods have been experimented with for gene therapy of lysosomal disorders. Several of these methods have proved efficient for the transfer of genetic material into deficient cells in culture and reconstitution of enzyme activity. However, the same methods applied to humans or animal models have been giving inconsistent results, the bases of which are not fully understood. A broader knowledge of disease pathogenesis, facilitated by available, faithful animal models of LSDs, coupled to the development of better gene transfer systems as well as the understanding of vector host interactions will make somatic gene therapy for these devastating and complex diseases the most suitable therapeutic approach.

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Introduction

Nature and Function of Lysosomal Hydrolases

The major site of compartmentalized digestion of intra- and extracellular macromolecules is the lysosomal system, a morphologically heterogeneous population of organelles present in virtually all animal cells with the exception of the erythrocytes [1]. With their content of

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Fax + 41 61 306 12 34
E-Mail karger@karger.ch
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Alessandra d'Azzo
Department of Genetics and Tumor Cell Biology, St. Jude Children's Research Hospital
332 North Lauderdale Street
Memphis, TN 38105 (USA)
Tel. +1 901 495 2698, Fax +1 901 526 2907, E-Mail alessandra.dazzo@stjude.org

Table 1. Lysosomal diseases

Disease	Enzyme deficiency	
Sphingolipidoses		
GM1 gangliosidosis	β-Galactosidase	230500
GM2 gangliosidosis		
Variant B or B1: Tay-Sachs disease	Hexosaminidase A	272800
Variant O: Sandhoff's disease	Hexosaminidase A and B	268800
Variant AB	GM2 activator protein	
Metachromatic leukodystrophy	Arylsulfatase A	250100
Krabbe disease	Galactosylceramidase	245200
Fabry disease	β-Galactosidase A	301500
Gaucher disease	β-Glucosidase	230800, 230900, 231000
Niemann-Pick A or B disease	Sphingomyelinase	257200
Farber's disease	Ceramidase	228000
Wolman's disease	Acid lipase	278000
Austin's disease	Multiple sulfatase deficiencies	272200
Mucopolysaccharidoses		
Type I: Hurler's syndrome (IH)	α-*L*-Iduronidase	252800
Scheie disease (IS)	α-*L*-Iduronidase	252800
Type II: Hunter disease	Iduronate-2-sulfate-sulfatase	309900
Type III: Sanfilippo disease		
Type III A	Heparane sulfamidase	252900
Type III B	N-Acetyl-α-glucosaminidase	252920
Type III C	Acetyl-CoA: α-glucosaminide-N-acetyltranferase	252930
Type III D	N-Acetylglucosamine-6-sulfate-sulfatase	252940
Type IV: Morquio disease		
Type IV A	N-Acetylgalactosamine-6-sulfate-sulfatase	253000
Type IV B	β-Galactosidase	253010
Type VI: Maroteaux-Lamy disease	Arysulfatase B	253200
Type VII: Sly disease	β-Glucuronidase	253220
Type IX	Hyaluronidase (HYAL1)	601492
Pycnodysostosis	Cathepsin K	265800, 601105
Glycoproteinoses		
Aspartylglucosaminuria	Aspartylglucosaminidase	208400
Fucosidosis	α-Fucosidase	230000
α-Mannosidosis	α-Mannosidase	248500
β-Mannosidosis	β-Mannosidase	248510
Schindler and Kanzaki diseases	α-N-Acetyl-galactosaminidase or α-galactosidase B	104170
Galactosialidosis	Protective protein/cathepsin A (PPCA)	256540
Mucolipidoses		
Type 1: Sialidosis	α-Neuraminidase	256550
Type II cell disease	N-Acetylglucosamine-1-phosphotransferase	252500, 252600, 252605
Type III Pseudo Hurler polydystrophy	N-Acetylglucosamine-1-phosphotransferase	252500, 252600, 252605
Type IV Mucolipidosis	Mucolipidin	252650, 605248
Glycogenosis type II		
Infantile (Pompe's disease) and adult forms	α-1,4-Glucosidase or acid maltase	232300
Ceroid lipofuscinoses		
Locus CLN1: infantile form (Santavuori-Haltia)	Palmitoyl protein thioesterase	256730
Locus CLN2: late infantile form (Jansky-Bielschowsky)	Tripeptidyl peptidase I	204500
Locus CLN3: juvenile (Batten)	CLN3 protein	204200
Locus CLN5: late infantile form (Finnish variant)	CLN5 protein	256731
Locus CLN6: late infantile form (variant)	?	601780
Locus CLN7: late infantile form (variant)	?	No OMIM
Locus CLN8: northern epilepsy	CLN8 protein	600143
Others		
Cystinosis	Cystinosin (cystine carrier)	219800, 219900, 219750
Sialic acid storage diseases (infantile form, Salla disease)	Sialin (sialic acid carrier)	269920, 604369, 604322
Niemann-Pick type C: NPC1 and NPC2	NPC1 protein, NPC2 (epidymal secretory protein)	257220, 601015

Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM

hydrolytic enzymes, lysosomes maintain cellular homeostasis by regulating the turnover of many cellular constituents; proteins, glycoproteins, nucleic acids, polysaccharides and glyco-, phospho- and neutral lipids are their natural substrates [1]. Most soluble enzyme precursors reach the lysosome by going across the secretory pathway to the trans Golgi network and the endosomal compartment. This tightly regulated process entails the acquisition and processing of the sugar chains, the synthesis of the M6P recognition marker, the segregation from secretory proteins, and final maturation and activation in endosomes/lysosomes. By default, a small percentage of the precursor proteins can also be recovered in the extracellular milieu, but these secreted forms retain the capacity to be reinternalized via receptor-mediated endocytosis, and to be efficiently targeted to the lysosome where they function normally [2–4].

In general, lysosomal enzymes are specific with respect to the chemical linkage and structure of the monomeric unit that they hydrolyze. This specificity is reflected in the wide range of glycosidases that represent one of the largest classes of lysosomal enzymes. Glycosidases bring about the catabolism of specific sugar chains on glycoconjugates in a step-wise and concerted fashion. If any one of these enzymes is deficient or functionally defective, the process of degradation is halted at the level of the missing enzyme. This block leads first to the progressive intralysosomal accumulation of partially degraded metabolites, and subsequently to the cellular and organ dysfunction associated with a lysosomal storage disease (LSD) [4, 5]. Not surprisingly, genetic lesions that result in faulty enzyme function are heterogeneous, given the numerous modifications that must occur with some precision between the site of synthesis of a lysosomal protein and its final sequestering and full activation within the lysosome. The genotype and familial history of the disease may eventually impact the response to treatment.

Lysosomal Storage Diseases

LSDs comprise a group of over 40 monogenic neurodegenerative disorders of metabolism, the majority of which are autosomal recessive. Their incidence as a group accounts for 1 in ~5,000 life births. With few exceptions, these disorders are caused by either single or multiple deficiency of glycosidases, and are classified, according to the type and site of the primary accumulated products, as mucopolysaccharidoses (MPS), mucolipidoses, glycoproteinoses, sphingolipidoses and others (table 1). Overall LSDs represent a great burden for society in terms of clinical and diagnostic care, and no curative therapies are available as yet for these diseases. However, the very nature of soluble lysosomal enzymes and their unique capacity to be transferred from one cell to neighboring cells at near or distant sites have made LSDs particularly amenable models for the development of therapeutic modalities. In principle, the enzyme deficiency of cells can be corrected when these cells take up the missing enzyme from exogenous sources (fig. 1).

Both severe, early onset forms and milder late onset variants are distinguished in most of the LSDs. This clinical heterogeneity often correlates with the residual amount of functional enzyme in lysosomes; this implies that even modest increases in enzyme activity, if they occur early in life and at the correct cellular site, might prevent/cure the disease. The fact that carriers are asymptomatic suggests that the full complement of enzyme activity is not needed to maintain a normal phenotype or to achieve therapy. Enzyme levels as low as 5–10% may be sufficient to prevent the occurrence of symptoms, although the threshold of enzyme able to cope with the metabolic need of any given cell may vary from individual to individual and may be influenced by genetic background or environmental factors [4, 6].

Clinically, LSDs are complex, systemic diseases that affect to different extents the visceral organs, the heart and skeletal muscles, the bones and cartilage and most importantly the central (CNS), and peripheral nervous systems [4, 7]. Common features include severe psychomotor delay, visceromegaly, growth retardation and early death. Neurological abnormalities, in particular, may alter dramatically disease prognosis and complicate the applicability of potential therapeutic modalities. These variations in disease penetrance for both the systemic and nervous systems likely reflect differences in the metabolic needs of individual cell types that, in turn, may depend on the selective nature of the primary defect. Although the common macroscopic evidence of LSD in tissues is the presence of vacuolated cells, the cellular and molecular consequences of the intralysosomal accumulation of various metabolites are largely unknown. Given the complexity of these disorders, this information will prove crucial in addressing the feasibility and limitations of therapy for neurodegenerative LSDs. Studies have now begun to emerge that will help to relate storage of potentially toxic metabolites to cell dysfunction and cell death [8, 9].

The large number of genetically engineered mouse models of LSDs that are currently available combined with spontaneously occurring animal models, is facilitating this endeavor. In most instances, these models resemble closely the corresponding human diseases and are

therefore becoming increasingly important for studies of the pathogenesis and treatment of these disorders [10–12].

Therapy for Lysosomal Storage Diseases

The mechanism of cross-correction has formed the basis of different therapies for LSDs such as enzyme replacement therapy (ERT), hematopoietic stem cell (HSC) transplantation, and gene therapy. The results of ERT in clinical trials of Gaucher disease [13, 14], Pompe disease [15], Fabry disease [16], and MPS I [17] as well as in animal models [18–25] have demonstrated that this procedure could be effective against some non-neuropathic LSDs. However, ERT alone is unlikely to ameliorate more severe neuropathic forms of LSDs, unless it is combined with other treatments [26]. The presence of the blood-brain barrier that effectively prevents most soluble molecules from entering the CNS [27] hampers the applicability of this approach for the cure of neuropathic patients.

Unlike ERT, HSC transplantation (using bone marrow (BM) or umbilical cord blood) could provide a permanent source of normal enzyme, since HSCs can in principle differentiate and repopulate target organs, including the CNS (fig. 2), and function as donors of the corrective enzyme to deficient cells [28–30]. Allogeneic BM transplantation (BMT) with marrow from HLA-compatible, partially mismatched, or unrelated donors has been used with increasing frequency to treat patients with LSDs. The outcome is largely contingent on the type and stage of the disease. In general, this procedure is relatively effective in alleviating visceral symptoms and in stabilizing bone lesions, provided that it is performed before the occurrence of major neurological damage [31–33]. However, diseases that have an early onset and involve predominantly the CNS respond poorly or not at all to BMT, albeit some variations in outcomes have been observed among disease subtypes [32, 34]. For example, the long-term effects of transplantation in patients with Hurler syndrome (α-iduronidase deficiency, MPS I) have been encouraging but by no means curative [35, 36]. Skeletal abnormalities were only minimally altered [37, 38] and psychomotor functions were at best stabilized [35, 38]. BMT appears to change the course of the disease in patients with Krabbe disease and metachromatic leukodystrophy (MLD), but only if performed prior to the occurrence of severe clinical signs [34, 39]. Dramatic resolution of the systemic pathology, improvement in bone disease and stabilization of neurocognitive functions have been seen in transplanted α-mannosidosis and fucosidosis patients [40–42]. In contrast, the outcome of treatment in aspartylglucosaminidase (AGA)-deficient patients has been disappointing [43] and the same holds true for GM1- and GM2-gangliosidoses [44].

The results of BMT in small and large animal models of LSDs seem to parallel that in human patients. The clinical responses vary depending on the disease subtypes and the age at treatment, especially with regard to the CNS disease [45, 46]. Thus, syngeneic BMT in neonatal β-glucuronidase-deficient (MPS VII) mice has proved more successful than in adult mice for ameliorating both visceral and bone pathology, and improving auditory function, even in the absence of myeloablation [47–49]. However, the procedure did not reverse storage in the brain or improve the behavioral deficits in these mice [47, 50, 51]. The outcome of BMT in the Niemann-Pick disease (NPD) and galactosialidosis mouse models has been surprisingly positive [52–54]. Even though the correction of the CNS pathology was only minimal, rescue of Purkinje cell loss was apparent in treated NPD mice. Encouraging results were also obtained in cats with α-mannosidosis and dogs with fucosidosis where a clear neurologic improvement and a reduction of storage in neurons were detected after transplantation of young pups [55, 56]. Among the gangliosidoses, BMT extended the lifespan, corrected the biochemical deficits in systemic tissues, and slowed the neurological deterioration in GM2-gangliosidosis mice, in spite of the persistence of neuronal pathology [57]. However, the same procedure applied to dogs with GM1-gangliosidosis or cats with GM2-gangliosidosis did not significantly reverse their pathology [46, 58].

Despite these variations in therapeutic outcome, the overall results suggest that BM-derived cells are capable of infiltrating the CNS and supplying normal enzyme to deficient cells, thereby restoring lysosomal function [30]. In general, the earlier in life that the transplant is performed, the better the clinical outcome. Response to BMT may also be influenced by: (1) the type and number of engrafted donor cells; (2) the biochemical and physical properties of the secreted, correcting enzyme; (3) the efficiency of secretion and extracellular stability of the correcting enzyme [59]; (4) the extent of uptake by target cells, and (5) the characteristics of the affected cells as well as the level of cell degeneration.

Some of these variables have been tested in proof-of-principle studies by using transgenic BM cells overexpressing the therapeutic enzyme under the control of an erythroid- or macrophage-specific promoter to correct the

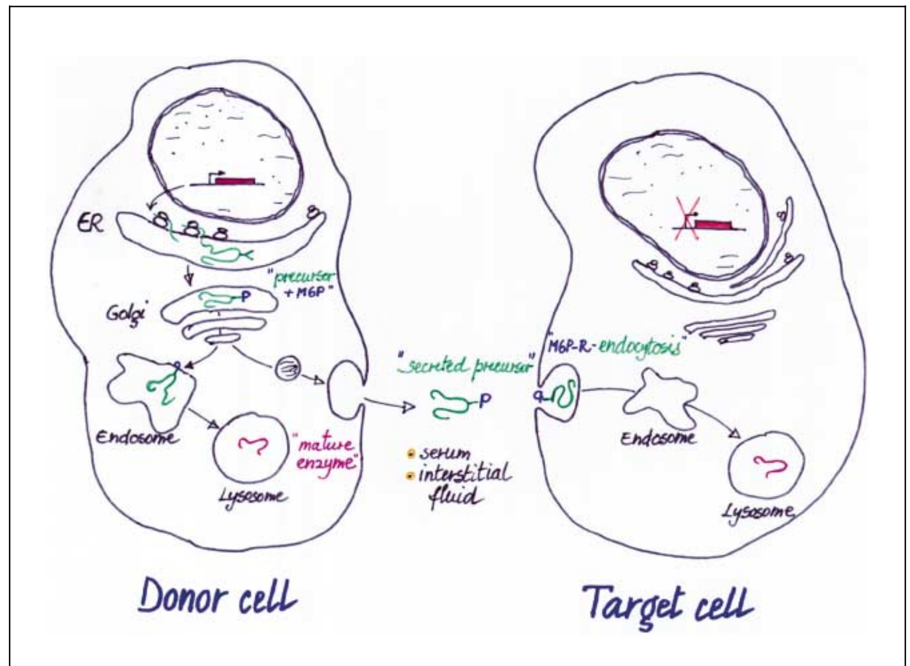

Fig. 1. Default pathway of lysosomal enzyme precursors. A small percentage of the soluble enzyme precursors is secreted into the extracellular space, but this secreted pool can be taken up via receptor-mediated endocytosis at near or distant sites and be routed to the lysosomes.

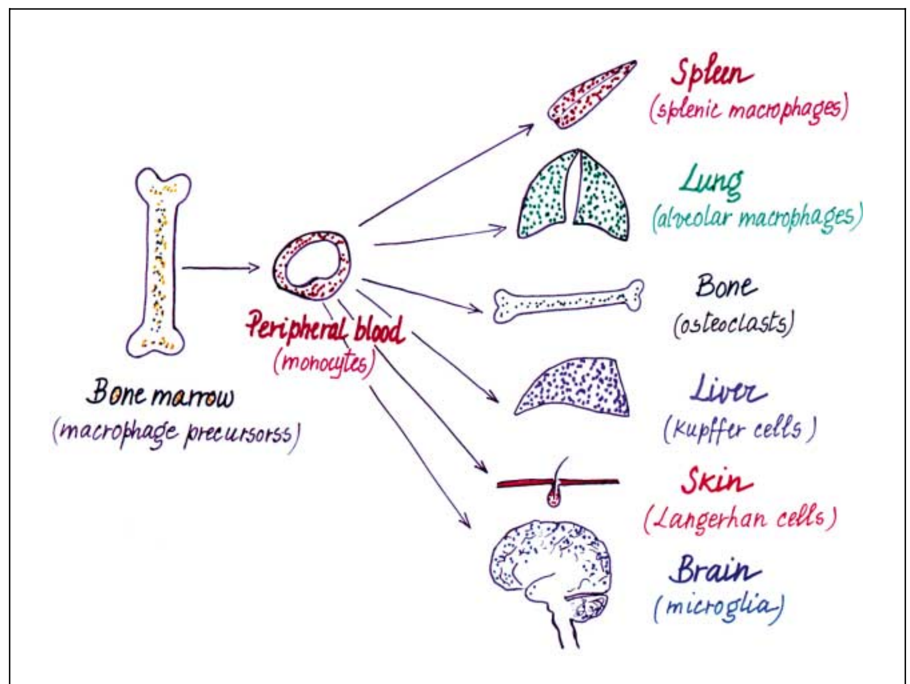

Fig. 2. BM-derived monocytes/macrophages. These cells present in the circulation or residing in different organs can be the continuous source of correcting enzyme in transplanted recipients.

disease phenotype in the galactosialidosis mouse model [52, 54]. This experimental approach is based on the hypothesis that higher levels of enzyme would provide effective treatment for the systemic and CNS disease in a shorter period of time. The results of these studies have validated this idea, and unequivocally demonstrated that homogeneous populations of hematopoietic cells overexpressing the therapeutic enzyme can effectively cross-correct affected cells and improve/delay the CNS pathology in both BMT and crossbreeding experiments.

Gene Transfer and Gene Therapy for Lysosomal Storage Diseases: Pros and Cons

Ex vivo Gene Transfer

The same advantages and hurdles associated with BMT can be applied to a gene therapy approach based on the use of genetically modified HSCs. This procedure, however, should overcome the main limitations of allogeneic BMT in humans: namely, the difficulty in finding HLA-matched donors, and the morbidity and mortality associated with irradiation, immunosuppression, and graft-versus-host disease. In fact, somatic gene therapy could become the preferred treatment for LSDs if the patient's own cells could be genetically modified in vitro or in vivo to constitutively express and secrete higher levels of the correcting enzyme than normal BM, and could become the source of the correcting enzyme in the patient. Both ex vivo and in vivo gene transfer methods have been tested for somatic gene therapy of LSDs. The range of delivery systems, starting with retroviral vectors, has expanded to include vectors based on adenovirus, adeno-associated virus, herpes simplex virus, lentivirus, and others, as well as non-viral systems. Several of these methods have proven efficient for the transfer of genetic material into deficient cells in culture and consequent restoration of the enzyme activity [60–71]. However, the same gene transfer systems applied to patients or affected animals have been hampered by inconsistent results, the bases for which are not yet fully understood.

Among the problems are the types of currently available vectors, the poor understanding of vector–host interactions, particularly in the context of a pathologic condition, the low transduction efficiency of pluripotent stem cells and the inability to achieve long-term expression of the correcting protein in vivo. Some of these difficulties could be circumvented by the use of improved viral vectors, virus purification conditions [72–74], transduction procedures, and viral packaging cell lines [75–80]. Moreover, the expansion, transduction, and selection of target cells, like BM stem cells, in vitro have been facilitated by the use of specific cell culture conditions and selectable markers [80a–c].

Retroviral vectors have so far been the most exploited gene transfer vehicles for therapy of LSDs. Those commonly used are the ecotropic and amphotropic murine leukemia viruses [81, 82]. These vectors have the advantage that they stably integrate at random sites into the host genome, affording long-term expression. However, they are limited in their use since they require dividing cells as a target; they are difficult to produce at high titer for in vivo application and, depending on their integration site, they can permanently alter expression of neighboring genes. Nevertheless, an ever-growing number of studies are reported in the literature that describe the use of retroviral-mediated gene therapy in animal models of LSDs. Transplantation of transduced syngeneic HSCs and BM macrophages in the MPS VII mice has resulted in increased enzyme activity and reduced lysosomal storage only in the liver and spleen, but neither brain nor bone pathology were improved by this procedure [51, 83, 84]. In contrast, the effects of a gene therapy approach in PD mice replicated those obtained with normal BM and consisted in prolonged life span, increased acid sphingomyelinase activity, reduced storage in both systemic organs and spinal cord neurons, and an increased number of Purkinje cell neurons [85]. Equally encouraging results were obtained in the mouse models of MLD and galactosialidosis using a murine stem cell virus-based retroviral vector [86, 86a]. Long-term expression of the enzyme and most notably transfer of arylsulfatase A to the brain were achieved in the MLD mice. In the protective protein/cathepsin A (PPCA)-deficient mice, complete correction of systemic pathology was accompanied by delayed Purkinje cell loss and functional amelioration of the neurological phenotype. In Fabry disease, mice transplanted with retrovirally transduced BM mononuclear cells increased α-galactosidase activity and decreased storage was observed in all organs with the exception of the brain [87].

The overall outcome of these studies points to the potential of retroviral-mediated gene transfer to BM stem cells for improving systemic disease in small laboratory animals, but again the efficacy of this procedure in treating CNS pathology needs careful evaluation. In addition, the same procedure applied to larger animal models has given inconsistent results. For example, dogs with fucosidosis (α-fucosidase deficiency) transplanted with retrovirally transduced allogeneic or autologous BM failed to engraft, although the transduction efficiency was high [88]. Similarly, in MPS VI cats that received genetically marked autologous BM or neonatal blood cells, the levels of arylsulfatase B remained low and no clinical improvement was noticed during the 2-year follow-up, despite evidence of long-term engraftment [89]. However, neonatal gene therapy in MPS VII dogs was successful in preventing the main clinical manifestations of the disease [89a]. Once more, the time of treatment seems to be the rate-limiting step of these therapeutic procedures especially if we attempt to prevent, delay progression or even reverse CNS pathology.

As alternative approaches to ex vivo gene therapy directed to the hematopoietic cells, investigators are also testing the use of other genetically modified cells and vectors for delivery of the correcting enzyme at target sites. For instance, retrovirus-transduced fibroblasts expressing β-glucuronidase, embedded into a collagen lattice have been implanted intraperitoneally into MPS VII mice or dogs. The enzyme secreted by these neo-organs cleared storage in the liver and spleen of MPS VII mice and reduced liver pathology in the dogs [90, 91]. However, for delivery of the therapeutic enzyme across the blood-brain barrier, the recombinant fibroblasts had to be directly implanted into the brain parenchyma. Although sustained β-glucuronidase expression and clearance of storage in neurons were observed, these effects were limited to cells in the proximity of the graft and the activity decayed after an approximately 1-month period [92]. Using this delivery system in normal dogs, expression of β-glucuronidase was detected for nearly a year following implantation into the omentum of six lattices containing retrovirally transduced fibroblasts [93]. In a similar approach, alginate microcapsules containing non-autologous fibroblasts have been implanted either intraperitoneally or in the brain of MPS VII mice [94, 95]. Again, the liver and spleen were readily cleared of storage and expressed significant levels of β-glucuronidase activity, but the enzyme elicited an antibody response that had to be controlled with immune suppressants to prolong the effect of the treatment [94]. Interestingly, reversal of histopathology in the brain was accompanied by improvement in the behavioral abnormalities for up to 2 months after treatment. Equally efficient for the short-term improvement in brain pathology were human amniotic epithelial cells marked with an adenoviral vector expressing β-glucuronidase and injected into the corpus striatum [96]. Since these cells are immunologically naïve, they could become an attractive system for local delivery of high quantities of a therapeutic enzyme, especially at sites that are difficult to reach, although issues concerning safety and long-term expression have still to be addressed.

Considering that the properties of donor cells may greatly influence the efficacy of cell-mediated therapy for neurologic LSDs, intracranial transplants of genetically modified neural stem cells or adult glial cells could potentially be a better choice for treating the CNS [97–99]. Their therapeutic potential has been tested in MPS VII and twitcher mice (model of Krabbe disease). In MPS VII newborns that received intraventricular transplant of a multipotent neural cell line expressing β-glucuronidase, donor cells appeared to engraft, become part of the brain microenvironment, and correct lysosomal storage in both neurons and glia [100]. Similar experiments performed in the twitcher model indicated that these neural progenitor cells could differentiate in several cell types including oligodendrocytes, the primary affected cells in this disease; however, correction of the demyelinating defect was only minimal [101]. Whether these transformed neural cell lines transplanted into the brain will eventually give rise to intracerebral tumors has not been tested as yet. In this respect, the encouraging, albeit short-term, results obtained with transduced primary human astrocytes engrafted into the mouse striatum point to this cell type as an alternative and perhaps safer system for treatment of the CNS [99].

The use of BM stem cells as the source of the enzyme for treatment of the CNS disease has recently been extended. It was shown that BM contains non-hematopoietic stem cells capable of differentiating into multiple mesenchymal cell lineages, including neural cells [102]. These cells, referred to as marrow stromal cells or mesenchymal stem cells, have several attractive characteristics: they are easy to isolate from BM, to expand in culture, and to transduce in vitro. They may, therefore, have advantages over HSCs for gene therapy.

All together, cell-mediated delivery methods have the potential to provide sustained expression of therapeutic proteins at target sites. On the other hand, with the exception of one study in normal dogs [93], the follow-up of treatment has been relatively short; hence no indication is there for the long-term engraftment of the exogenous cells, expression of the therapeutic enzyme and the potential side effects. Moreover, local administration of neo-organs or modified cells does not fulfill the need of generalized correction of lysosomal storage unless it is coupled to a more systemic therapy.

In spite of the limitations encountered so far, the ex vivo studies in animal models have helped to set the stage for trials of human stem cell gene therapy in patients with Gaucher disease [103–105]. HSCs from patients' peripheral blood or BM were transduced with a retrovirus expressing the human glucocerebrosidase cDNA and infused into non-ablated recipients. Although the transduction efficiency was low, gene-marked cells persisted for ~ 3 months, but the number of corrected cells was too low to afford any increase in enzyme activity and therapeutic benefit. To circumvent the problems of low transduction efficiency and expression, alternative gene therapy approaches are being developed that make use of vectors based on lentiviruses, including the human immunodeficiency virus type-1 (HIV-1) [106]. These vectors have a

broader host range than retroviral vectors since they transduce both dividing and non-dividing cells and have been shown to effectively target human CD34+ cells [107].

In vivo Gene Transfer

Direct delivery of recombinant viral vectors into affected recipients would obviate the potential problems associated with in vitro manipulation of autologous cells and shorten the time that treatment can be initiated. Once more, in order to be effective these gene transfer vectors should be made at sufficiently high titer, should transduce a wide range of cells, be stable to achieve wide spreading, give sustained long-term expression and secretion of the correcting enzyme, and, most importantly, be safe. If all these parameters are met, the in vivo methods could be chosen as alternative or complementary systems to ex vivo therapy for correction of both systemic and CNS disease in LSDs. Significant progress has been made recently using adenovirus and adeno-associated virus vectors for in vivo gene transfer in animal models.

Adenoviral vectors have several advantages over retroviruses: they infect both dividing and non-dividing cells; they can accommodate larger inserts of exogenous DNA, and they can be produced at high titer [108, 109]. The downsides of these vectors have been their potential cytotoxicity, the high immunogenicity of viral proteins [110, 111] and in turn the transient expression of the therapeutic enzyme that limits its effects. Nevertheless, a few groups have now reported the successful use of this transfer system for the short-term correction of diseases that are predominantly systemic. A recombinant adenovirus expressing the human α-galactosidase A was injected intravenously into Fabry mice. Enzyme activity considerably higher than normal values was measured in all systemic organs 3 days after injection but declined rapidly during the following 12 days. Remarkably, however, the high level of enzyme in organs and plasma was sufficient to substantially improve the pathology for up to 6 months after treatment [112]. Similar studies performed in mice with Pompe disease demonstrated that a single injection of a modified adenoviral vector expressing α-glucosidase resulted in efficient hepatic transduction, increased enzyme secretion in plasma and systemic correction of both skeletal and cardiac muscles, the primary organs affected by the disease [113]. In Tay Sachs mice intravenous injection of a recombinant adenovirus co-expressing the hexosaminidase subunits resulted in high transduction of the liver, massive secretion of the enzyme in serum and restoration of enzyme activity in all peripheral organs [114]. Thus, albeit transient, adenovirus-mediated expression of the therapeutic enzyme is high enough to provide a prolonged effect, especially if immune suppressive agents are simultaneously administered [115].

For the treatment of the CNS pathology, a number of reports have documented the combined use of recombinant adenoviral vectors for intravenous injection as well as direct injection into the brain. In the aspartylglucosaminuria mouse model, correction of the liver pathology was associated with sustained AGA expression in the ependymal cells lining the ventricles and diffusion of AGA into the neighboring neurons with partial amelioration of the pathology [116]. In the MPS VII mice, intravenous injection coupled to systemic treatment with immune-suppressive antibody resulted in normal β-glucuronidase activity in systemic organs for up to 16 weeks after treatment. Impressive was the reduction of pathology in the brain, even if the number of cells expressing the β-glucuronidase mRNA were only a few [115, 117, 118]. These are clear examples of the remarkable bystander effect that can be reached if the expression and secretion of the correcting enzyme is high and the enzyme is readily taken up. This of course will entirely depend on the physiological and biochemical characteristics of both the disease and the enzyme itself. For example, direct intraventricular injection of a recombinant adenovirus encoding galactocerebrosidase in twitcher mice demonstrated only slight improvements in motor functions, body weight and twitching but only when treatment was initiated at postnatal day 0 and not at postnatal day 15 [119].

Moreover, it is clear that the therapeutic potential of recombinant adenovirus vectors for the treatment of LSDs and other clinical conditions in humans cannot be fully exploited unless we are confident that their limitations can be completely overcome. Recent results with modified adenovirus vectors in which nearly all adenoviral sequences have been removed (high-capacity 'gutless' vectors) show promise for reduced toxicity and long-term expression [120, 121].

Adeno-associated virus (AAV) is a replication-deficient human parvovirus that has the capacity to transduce with equal efficiency both dividing and non-dividing cells, including neurons [122]. The most attractive features of this virus that encourage the use of AAV-based vectors in human clinical trials [123] are: (1) the capacity of latent infection via integration into the host genome; (2) the lack of the pathogenic or inflammatory side effects, and (3) the long-term expression of the transgene [124–126]. In spite of their small insert capacity, recent improvements in the production of high-titer, purified re-

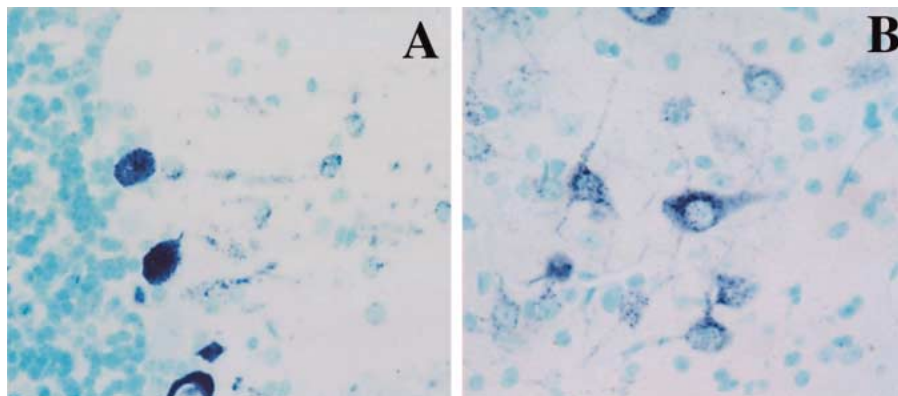

Fig. 3. Expression of human PPCA in AAV injected galactosialidosis mice. Histological sections of the cerebellum for galactosialidosis mice injected with a recombinant AAV vector expressing human PPCA were immunostained with a mono specific anti-PPCA antibody. Sustained expression of PPCA was detected in numerous Purkinje cells (**A**) and in neurons of the deep cerebellar nucleus (**B**).

combinant AAV vector stocks have increased the number of applications of AAV as a gene transfer system. Most of the initial experiments with AAV vectors were performed in the MPS VII mouse model. An intramuscular administration of a recombinant AAV vector encoding β-glucuronidase to neonatal or adult mice resulted in persistent transgene expression in the injected organ and partial amelioration of lysosomal storage in the liver and spleen. The latter was likely attributable to virus dissemination through the blood stream rather than secretion of the enzyme from infected muscle [127, 128]. In contrast, a widespread improvement in the phenotype was obtained by injecting a β-glucuronidase expressing AAV vector in newborn mice (1–2 days old). This early treatment gave rise to high levels of enzyme activity and prevented/reduced lysosomal storage in most organs including the brain throughout the 4-month duration of the experiment [129].

The AAV gene transfer system has also been effective for treating the CNS disease. Again, most of the studies have been done in neonatal and adult MPS VII mice and in most cases with a limited follow-up after treatment. A single intracranial injection of a β-glucuronidase expressing AAV vector into the striatum of a severely affected adult mice was enough to revert pathological lesions in a significant portion of the brain. The effect persisted for up to 16 weeks and spreading of enzyme-positive cells in the contralateral hemisphere was indicative of a retrograde transport of the virus [130]. A similar procedure applied to newborn mice was successful not only in preventing or reducing neuronal storage but also in improving cognitive function without detectable side effects [131]. Intrathecal injection into the cerebral spinal fluid has also been experimented with as a less invasive route of administration of the virus to mutant mice [132]. A reduction of storage in some neurons and increased β-glucuronidase activity in the brain were obtained with this procedure, although there was no indication of the extent of correction of the brain pathology.

The encouraging outcome of the studies in the MPS VII model have prompted the use of this delivery system in other LSDs. Jung et al. [133] have injected a recombinant AAV expressing human α-galactosidase in the portal vein of Fabry mice. Six months after treatment the α-gal A activity was about 10–15% of control values in the liver and other systemic organs, and was accompanied by a considerable reduction in glycolipid storage. Preliminary studies on the San Filippo B mice injected intracranially demonstrated persistent expression of the correcting enzyme and a reduction in storage [134]. A single injection of an AAV vector encoding PPCA directly into the cerebellum of galactosialidosis mice resulted in sustained expression of the enzyme in several Purkinje cells and neurons of the deep cerebellar nucleus (fig. 3) with consequent delay in Purkinje cell loss characteristic of this model [135]. A long-term follow-up of the treated mice demonstrated a clear improvement in their coordination skills and lack of tremor associated with the ataxic phenotype [Cunningham, Smeyne and d'Azzo, personal communication]. On the other hand, a similar vector expressing human galactocerebrosidase injected intravenously and/or intracranially into neonatal Twitcher mice did not improve their clinical condition [136], although this may be due to the incapacity of the human galactocerebrosidase to be properly processed in mouse cells [Wenger D., personal commun.]. The overall lesson from these studies is that recombinant AAV vectors may not be equally effective in correcting different LSDs and underscore the need of achieving a better understanding of virus-host interaction as well as disease pathogenesis. In addition a

careful evaluation of the long-term effects of treatment is mandatory in view of the recent findings that a large proportion of MPS VII mice injected with a recombinant AAV vector shortly after birth develop hepatic tumors 1–1.5 year after treatment [137].

Other viral vectors are also being considered. A few interesting studies have been pursued recently that are based on the use of lentivirus vectors, including human, and feline immunodeficiency viruses (HIV and FIV) for gene therapy of LSDs in animal models. Similar to retroviruses, lentiviral vectors integrate stably into the host genome, but have the advantage of infecting both dividing and non-dividing or terminally differentiated cells and accommodating large insert size [81, 138]. In a difficult to treat disease like MLD, Consiglio et al. [139] have achieved sustained arylsulfatase A expression and effective rescue of hippocampal neurons following injection into the affected brains of a HIV-1-based vector expressing human ASA. Although the MLD mice were injected at adult age (5 months), the treatment was apparently sufficient for the long-term correction of the injected hemisphere and protection from progressive deterioration of hippocampal-related learning ability. Very encouraging results were also obtained in adult MPS VII mice using a similar lentiviral vector [140]. The recombinant virus was delivered by either single intra-striatum injection or multiple injections in the cerebral hemispheres and the cerebellum. Clearance of storage was observed throughout the brain suggesting that reversal of advanced brain lesion can be achieved if a suitable delivery system is applied. Two-month-old MPS VII mice have also been injected intravenously with a single dose of a FIV-based vector expressing β-glucuronidase. Sustained β-glucuronidase activity was detected in several tissues with a parallel reduction in pathological signs in the liver and spleen. These results indicate that the hepatocytes, when stably transduced with lentivirus-based vectors, can serve as a reservoir of the correcting enzyme for the long-term therapy.

Obviously the main concern raised by the use of this type of vector is safety. As is the case for other gene transfer systems, HIV-based vectors have been stripped of most of the HIV genes whose functions are supplied in trans [141]. Nonetheless, potential risks, albeit remote, of recombination events that would reconstitute the original viral genome must be meticulously controlled and more experimental examples must be evaluated.

Despite significant accomplishments, until vector systems are developed that provide high transduction efficiency, low immunogenicity, long-term protein expression in the appropriate target cells, and correction of difficult to reach organs, the full potential of this approach will not be realized. New and improved methods for delivering the therapeutic genes to affected sites are being developed. The use of hybrid or chimeric vectors, incorporating different viral elements have been shown to improve both transduction of recipient cells and transgene expression [142]. The induction of systemic hyperosmolarity to temporarily open the blood-brain barrier have facilitated the penetration of vector across the ependymal cell layer into the subependymal region and improved spreading of β-glucuronidase-expressing cells [143]. Intravenous or direct brain injection of an adenoviral vector that encodes therapeutic β-glucuronidase fused to the protein transduction peptide of the HIV Tat have resulted in improved bio-distribution of the secreted enzyme in both visceral organs and the brain [144]. Finally, the testing of different viral serotypes may determine the choice of vectors for transfer of the correcting enzyme to different affected organs [145, 146].

Conclusions

A critical look at the myriad of gene therapy studies that are now facilitated by the availability of faithful animal models in which to implement therapeutic modalities should enable us to assess what is conceivable and what is not for the treatment of lysosomal disorders. How far we can go with the available systems and what needs to be improved. Behind the relatively simple concept of cross-correction that makes LSDs particularly amenable models for gene therapy trials lays the complexity and diversity of these diseases that must be addressed and carefully evaluated, since they are likely to influence the response to treatment. This underlies the continuing need for therapeutic studies to be guided by fundamental research into the molecular and cellular mechanisms of disease. It is clear that many limitations and pitfalls still need to be overcome in order to make the transition to the clinic an educated and judicious approach.

However, neither mice nor other larger animal models are humans. Thus, prior to clinical trials, it is important to come up with better methods to assess preclinical results. These include the large scale production, purification and quality control of viral vector stocks, the correct dose and route of administration, the evaluation of the efficacy of gene transfer in terms of the number of transduced cells present in target tissues, and the critical documentation of any clinical improvement. Regardless of the delivery sys-

tem, some diseases may be more resilient to treatment than others. In these instances we might approach therapy from the standpoint of the accumulated products rather the enzyme deficiency. Inhibition of substrate synthesis as opposed to supplementation of the missing enzyme has been experimented with for the treatment of glycolipid storage diseases with promising results [147, 148]. Combining substrate inhibitors and BMT was found to be synergistic in correcting pathological signs in the Sandhoff mice [149]. Last but not least, early treatment may be the only approach for early-onset patients who are often devoid of residual enzyme. In these cases therapeutic intervention in utero could prevent the appearance of any clinical symptoms and obviate the problems associate with an immune response [150]. A few experimental trials have been attempted that, although negative, are helping to set the stage for the use of this approach in both small and large animal models of LSDs [151, 152]. Ultimately the combination of different therapies may be the method of choice for neurological LSDs that require both systemic and CNS correction.

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Gene Therapy for Chronic Granulomatous Disease

W. Scott Goebel   Mary C. Dinauer

Herman B Wells Center for Pediatric Research and Department of Pediatrics (Hematology/Oncology),
James Whitcomb Riley Hospital for Children, Indiana University School of Medicine, Indianapolis, Ind., USA

Key Words
Gene therapy · Chronic granulomatous disease · Phagocytes · NADPH oxidase

Abstract
Identification of gene mutations responsible for leukocyte dysfunction along with the application of gene transfer technology has made genetic correction of such disorders possible. Much of the research into molecular therapy for inherited disorders of phagocytes has been focused on chronic granulomatous disease (CGD). CGD results from mutations in any one of the four genes encoding essential subunits of respiratory burst NADPH oxidase, the enzyme complex required for the production of reactive oxygen intermediates in phagocytes. The absence of phagocyte oxidants results in a predisposition to recurrent bacterial and fungal infections and inflammatory granulomas in CGD patients, associated with significant morbidity and mortality. Allogeneic bone marrow transplantation can cure CGD, but transplant-related toxicity and the limited availability of matched donors have restricted its wider application. Because the gene defects causing CGD are known, and CGD is a stem cell disorder treatable by marrow transplantation, CGD has emerged as a promising disease for somatic gene therapy targeted at the hematopoietic system. Multiple reports have demonstrated the reconstitution of NADPH oxidase activity by gene transfer to human CGD marrow and cell lines cultured in vitro. CGD mouse models have been developed by gene disruption, and preclinical studies on these animals using recombinant retroviral vectors have demonstrated reconstitution of functionally normal neutrophils and increased resistance to pathogens such as *Aspergillus fumigatus, Burkholderia cepacia* and *Staphylococcus aureus*. Although the results of these murine studies are encouraging, human phase-I clinical studies in CGD patients have yet to produce clinically beneficial numbers of corrected neutrophils for extended periods. Efforts to improve gene transfer efficiency into human hematopoietic stem cells and to increase engraftment of transduced stem cells are ongoing.

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Introduction

Chronic granulomatous disease (CGD) is a rare inherited disorder in which the leukocyte respiratory burst is absent or markedly deficient [1, 2] resulting in inadequate generation of highly reactive oxidants (e.g. superoxide, hydrogen peroxide and hypochlorous acid) necessary for microbicidal activity within the phagosome. As a consequence, CGD patients suffer from recurrent and often life-threatening bacterial and fungal infections. CGD oc-

KARGER
Fax + 41 61 306 12 34
E-Mail karger@karger.ch
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Mary C. Dinauer, MD, PhD
Cancer Research Institute R4, Indiana University School of Medicine
1044 W. Walnut Street
Indianapolis, IN 46202 (USA)
Tel. +1 317 274 8645, Fax +1 317 274 8679, E-Mail mdinauer@iupui.edu

Table 1. Classification of chronic granulomatous disease

Protein affected	Gene locus	Subtype[1]	Flavocytochrome *b*558 spectrum	NBT assay % positive	Frequency of cases, %
gp91*phox*	Xp21.1	$X91^u$	0	0	57
		$X91^-$	Low	80–100 (weak)	3
		$X91^+$	N	0	2
p22*phox*	16q24	$A22^u$	0	0	5
		$A22^+$	N	0	1
p47*phox*	7q11.23	$A47^u$	N	0	27
p67*phox*	1q25	$A67^u$	N	0	5

N = Normal; NBT = nitroblue tetrazolium assay.

1 In this classification, the first letter represents the mode of inheritance (X = X-linked or A = autosomal recessive). The number indicates the *phox* (for *ph*agocyte *ox*idase) component that is genetically affected. The superscript symbols indicate whether the level of the affected protein is undetectable (u), diminished (–), or normal (+) as measured by immunoblot analysis. $X91^+$ and $A22^+$ represent cross-reactive but defective enzymes.

curs in approximately 1 in 250,000 individuals [3], and is caused by a mutation in 1 of the 4 essential subunits of the NADPH oxidase complex (table 1) [1, 2, 4, 5]. Approximately two thirds of CGD cases are X-linked (X-CGD), and result from mutations in the gene encoding gp91*phox* (for *ph*agocyte *ox*idase), which along with p22*phox* constitutes the flavocytochrome *b*558, a plasma membrane heterodimer which is the redox center of the oxidase. The non-X-linked forms of CGD are autosomal recessive in inheritance. Only 5% of CGD cases are caused by mutations in the gene encoding p22*phox*, whereas the remainder of CGD cases involve mutations in either p67*phox* or p47*phox*, two cytoplasmic proteins that translocate to the membrane to interact with the cytochrome, forming the active oxidase complex upon phagocyte stimulation.

Because CGD is a hematopoietic cell defect, the disorder can be cured with allogeneic bone marrow transplantation [6–10]. However, because of the limited availability of matched donors, the regimen-related toxicity of the transplant, and post-transplant complications such as graft-versus-host disease, allogeneic marrow transplantation is not commonly employed to treat CGD. Because CGD is due to a single gene defect in hematopoietic stem cells, and mouse models of CGD have been developed which closely resemble the human disease, CGD has become an attractive target disease for hematopoietic cell gene replacement therapy [11], which could theoretically provide sufficient NADPH oxidase-reconstituted neutrophils to overcome, or at least ameliorate, the infectious and granulomatous complications of CGD. Correction of a minority of leukocytes is likely to lead to clinical improvement, since (1) partial chimerism following allogeneic marrow transplantation has been beneficial [6–9], and (2) female carriers of X-CGD with as few as 5–10% oxidase-positive neutrophils often have few or no symptoms [12–15]. The level of superoxide production within individual cells also appears to be important, since 'variant' X-CGD patients with low levels of residual NADPH oxidase activity can have recurrent infections [16]; partial correction of oxidase activity may be insufficient for microbicidal function.

Clinical Manifestations and Current Therapy

CGD patients typically develop recurrent bacterial and fungal infections beginning in early childhood [17–19]. CGD patients are susceptible to a wide variety of pathogens, but *Staphylococcus aureus, Aspergillus* species, and gram-negative enteric bacilli including *Serratia marcescens, Salmonella* species and *Burkholderia* (formerly *Pseudomonas*) *cepacia* are most problematic. Many of these organisms contain catalase, which depletes the phagosome of microbe-generated hydrogen peroxide that CGD neutrophils could otherwise use to kill ingested organisms. Frequent sites of infection include the skin, lymph nodes, bones and respiratory tract. The liver and lungs are not infrequently sites of abscesses and fungal infections. Stomatitis and severe gingivitis are common among CGD patients. The distinctive hallmark of CGD is the formation of chronic inflammatory granulomas, which can obstruct ureters and bowel or cause colitis/

enteritis. Granulomas may result from active infection, but these lesions are often sterile. Hence, it is postulated that chronic inflammatory lesions can result from the incomplete degradation of cellular debris which accumulates in the absence of respiratory burst-derived oxidants, or a dysregulated inflammatory response [17, 20–22].

The prognosis for CGD patients has improved greatly in the past two decades. Almost all patients with CGD died in early childhood when the disease was first described in the 1950s. Currently, infectious prophylaxis with trimethoprim-sulfamethoxazole and the aggressive treatment of acute infections has improved survival [23]. Subcutaneous injections of interferon-γ three times a week has greatly reduced the number of life-threatening infections for many patients [24]. Granulomas may respond to low-dose steroid therapy [25], but some require surgical resection. Morbidity due to infection or granulomatous complications unfortunately remains significant, particularly for patients with X-CGD [26]. The overall mortality has been estimated to be approximately 5%/year for X-CGD patients, and 2%/year for patients with autosomal recessive forms of CGD. This difference in survival is thought to be due to small amounts of superoxide production in the autosomal recessive subgroup of patients [3].

Gene Therapy for CGD

Recombinant retroviral vectors were first shown to be capable of transferring functional genes into murine bone marrow cells in early 1980s [27]. Gene transfer into human hematopoietic stem cells, however, has proved more difficult than originally anticipated. While efficient retroviral transduction of marrow-repopulating murine hematopoietic stem cells has been achieved, large animal and human studies have been hampered by low rates of gene transfer [28]. Reasons for poor gene transfer efficiency include the inability of retroviral vectors to integrate into quiescent hematopoietic stem cells [29], and a paucity of retrovirus receptors on primitive hematopoietic stem cells [30]. Several laboratories have improved stem cell transduction in large animal models, due to optimization of cytokine cocktails used in the in vitro transduction process, alternative retroviral envelopes, and co-localization of retroviruses and target cells via a fibronectin fragment to increase the efficiency of viral transduction [31–34]. These improvements have led to gene marking of up to 90% of human stem cells in some NOD/SCID mouse models [35–37]. In addition, small studies utilizing sub-myeloablative conditioning (320–500 cGy radiation) in rhesus macaques have reported detection of up to 15% gene-marked leukocytes as long as 33 weeks after transplantation in some animals, suggesting that low levels of gene correction may be feasible while reducing transplant-related toxicity [38, 39].

Preclinical in vitro Studies

The reconstitution of respiratory burst oxidase activity in human CGD leukocytes cultured in vitro using gene transfer technology has now been well established. Epstein-Barr virus (EBV)-transformed B-cell lines from CGD patients have been used as a model system by a number of groups. These cells express small amounts of the NADPH oxidase proteins and can produce low levels of superoxide, although the physiologic significance of B-cell superoxide generation is unclear. Respiratory burst activity can be restored to EBV-transformed B-cell lines derived from CGD patients in all 4 genetic subgroups using retroviral and/or plasmid-based vectors containing the appropriate cDNA [40–46]. Other studies have used a human myeloid X-CGD cell line developed by gene targeting, in which gp91*phox* expression and superoxide formation in retrovirus-reconstituted cells was quantitatively evaluated [47–50]. Expression of even modest amounts of recombinant gp91*phox* using either plasmid-based or retroviral vectors generated high levels of superoxide, suggesting that the flavocytochrome *b*558 complex is normally present in excess. Retroviral-mediated gene transfer to myeloid progenitors from peripheral blood or bone marrow obtained from CGD patients has also been successful. Functional expression of the proviral transcripts was documented in transduced granulocyte-monocyte cells differentiated in vitro using a sensitive chemiluminescence assay to monitor reconstitution of the NADPH oxidase, although the relative level of enzyme activity was not measured directly [43, 44, 51, 52]. Recently, however, transduction of up to 80% of CD34+ marrow cells from an X-CGD patient with a murine stem cell virus-based retrovirus containing gp91*phox* was demonstrated, with the resulting phagocyte superoxide levels in the transduced cells being 68.9% of normal levels [53].

In addition, reports exploring the potential of alternative vector systems for the correction of CGD have begun to appear. One study utilizing an HIV-1-based lentiviral vector containing gp91*phox* reported transduction of up to 63% of cells from a human X-CGD cell line, with mean superoxide production of in vitro differentiated cells measured at about one third of that of wild-type cells [54]. Very recently, Roesler et al. [55] reported correction of

superoxide production to 53% of normal in human CD34+ peripheral blood stem cells from human X-CGD patients transduced with a third generation, self-inactivating lentiviral vector containing gp91^{phox}, compared to 163% correction with a retroviral vector. The lentivirus-transduced stem cells, however, produced greater levels of gene-corrected neutrophils than the retrovirus-transduced stem cells when transplanted into immunodeficient NOD/SCID mice (20 vs. 2.4%), suggesting that lentiviral vectors may transduce the primitive stem cells capable of repopulating NOD/SCID mice better than retroviral vectors.

Preclinical Studies in Murine CGD

Retroviral-mediated gene transfer into bone marrow cells can correct respiratory burst oxidase activity in phagocytes in vivo and improve the defect in host defense against bacterial and fungal pathogens in murine models of CGD [15, 56, 57]. These studies were among the first to show the efficacy of gene therapy in improving the clinical symptoms of an inherited disorder, using an animal model that recapitulates the human disease.

In investigations using the gp91$^{phox-/-}$ (X-CGD) mouse, bone marrow cells were transduced with a murine stem cell virus-based retrovirus containing the murine gp91^{phox} cDNA [49, 58, 59] and transplanted into lethally irradiated (1,100 cGy, given in 2 doses) syngeneic X-CGD recipients [56, 57]. NAPDH oxidase activity was detected in 50–80% of circulating neutrophils by nitroblue tetrazolium testing 12–14 weeks after transplantation, which persisted for at least 18 months; gene-corrected neutrophils were also maintained in secondary recipients. These results demonstrate that this retroviral construct drives long-term expression of gp91^{phox} in neutrophils in vivo, and that long-term reconstituting hematopoietic stem cells were successfully transduced. Although neutrophil expression of vector-encoded gp91^{phox} protein was less than 10% of wild-type, superoxide generation was about one third that of wild-type mouse neutrophils. No adverse consequences to the long-term, constitutive expression of these levels of recombinant gp91^{phox} in marrow cells was observed [56, 57].

Even this level of NADPH oxidase function improved resistance of X-CGD mice to respiratory challenge with *Aspergillus fumigatus*. Pneumonia was prevented in gene-corrected X-CGD mice with restoration of partial enzyme activity to $\sim$50% of circulating neutrophils challenged with intratracheal *A. fumigatus* [56]. In X-CGD mice transplanted with mixtures of wild-type and X-CGD marrow, at least 5% wild-type neutrophils were required for protection against *A. fumigatus* challenge [56]. In 'gene therapy chimera' mice, generated by transplanting X-CGD mice with mixtures of retrovirus-transduced and mock-transduced X-CGD marrow, $\geq$11% gene-corrected neutrophils prevented the development of lung disease in 6/6 mice challenged with 150 intratracheal *A. fumigatus* spores, whereas 5–10% gene-corrected neutrophils prevented the development of lung disease in 2/6 mice [60]. Taken together, these studies suggest that partial reconstitution of NADPH oxidase activity after retroviral gene transfer can improve host defense in X-CGD, if a sufficient number of cells have enzyme activity.

In attempts to reduce the toxicity of the conditioning regimen for the transplantation of gene therapy-manipulated marrow, the use of low-dose radiation conditioning combined with larger doses of transplanted marrow cells was recently investigated [61]. In 160-cGy-conditioned X-CGD hosts transplanted with 20×10^6 transduced marrow cells, up to $\sim$10% gene-corrected neutrophils were observed 6 months after transplantation; these levels were stable for at least 12 months after transplantation in many recipients. These demonstrate that durable gene correction can be achieved using sub-myeloablative conditioning in a murine hematopoietic disease model, although the levels of gene-corrected cells are much lower than in recipients conditioned with 1,100 cGy.

The clinical benefit from retroviral-mediated gene transfer has also been studied in bacterial infection in p47^{phox}-deficient mice. In one study, recipient p47$^{phox-/-}$ animals received a sub-lethal dose of radiation (5 Gy) prior to transplantation of transduced marrow [15]. One month after transplantation of p47$^{phox-/-}$ bone marrow cells transduced with a retroviral vector for p47^{phox} expression, the percentage of superoxide-generating peripheral blood neutrophils ranged from 8.4 to 17.3% in individual mice (mean 12.3 $\pm$ 0.9%). Chimerism decreased to 2.6 $\pm$ 1.0% (range 0.8–9.9%) NADPH oxidase-positive cells 14 weeks after transplantation, and continued to fall thereafter. Oxidase activity in individual neutrophils appeared to be similar to wild-type cells. Following intraperitoneal challenge with a dose of *Burkholderia cepacia* that was lethal in 100% of untreated p47$^{phox-/-}$ mice, gene therapy-treated mice had significantly prolonged survival, and 2 of 9 mice studied apparently had spontaneous resolution of infection. Wild-type mice had no mortality even with a two-log higher dose of *B. cepacia*.

The above data demonstrate improvement in the host defenses of gene-corrected p47$^{phox-/-}$ mice, even with correction of a limited number of neutrophils. However, as suggested by the above study in the gp91$^{phox-/-}$ mice, cor-

rection of NADPH oxidase in greater than 5% of cells is likely to be required for more complete restoration of host defense. At least 30% gene-corrected neutrophils were required to prevent mortality in gp91$^{phox-/-}$ mice challenged with a different *B. cepacia* isolate, and the presence of even up to 25% wild-type neutrophils failed to prevent the formation of abscesses following challenge with *Staphylococcus aureus* [60]. As noted above, correction of >10% or more neutrophils was sufficient to prevent pulmonary aspergillosis in murine X-CGD. Overall, these observations are consistent with those in human X-CGD carrier females, in which some women with low levels of functional neutrophils (5–20%) suffer from recurrent bacterial infections typical of X-CGD patients, but *Aspergillus* infections are not seen [12–15].

The recent development of a model of cutaneous granuloma formation in murine X-CGD may prove to be useful for studying the benefits of gene therapy on the granulomatous complications of CGD [22]. X-CGD mice injected subcutaneously with sterilized *A. fumigatus* hyphae displayed a significant acute (3 days after injection) and chronic (30 days) inflammatory response compared to wild-type and X-CGD carrier female mice. All of the X-CGD mice also developed palpable granulomas by 30 days after injection; histologic examination of the injection site demonstrated granuloma formation, characterized by the infiltration of neutrophils and chronic inflammatory cells. We are currently using this model to study whether low levels of gene-corrected neutrophils in X-CGD mice can prevent the exaggerated inflammatory response and granuloma formation observed in X-CGD mice following subcutaneous challenge with sterilized *A. fumigatus* hyphae.

Phase-I Clinical Trials

A limited number of human clinical trials for gene therapy in CGD have been performed. Malech et al. [62] have conducted two phase-I clinical trials, using CD34+ stem cells from cytokine-mobilized peripheral blood as targets for retroviral-mediated gene transfer. In a completed trial involving 5 patients with the p47^{phox}-deficient form of CGD, mobilized peripheral blood CD34+ cells were collected by apheresis, transduced with a retroviral vector containing the p47^{phox} cDNA over a 3-day period ex vivo, and then transfused back into the patient [62]. Restoration of NADPH oxidase activity to 6–29% of granulocyte colony-forming cells was observed following in vitro culture of the transduced CD34+ cells. A much lower percentage of gene-corrected peripheral blood neutrophils was seen in vivo following transplantation of transduced cells, however. Using a sensitive flow cytometric assay of respiratory burst activity (dihydrorhodamine assay), oxidase-positive neutrophils were first detected approximately 3 weeks after transfusion, and persisted for several months. The peak percentage of corrected neutrophils, however, represented only 0.004–0.05% of the circulating neutrophils. This low percentage reflects the inefficiency of retroviral-mediated gene transfer into human hematopoietic cells, and the fact that patients received no marrow conditioning prior to reinfusion of transduced cells.

The same group subsequently used a modified transduction protocol for a phase-I study in X-CGD patients [63]. Retroviral transfer of the gp91^{phox} gene into autologous peripheral blood CD34+ cells was assisted by use of a fibronectin fragment [34] in the presence of a cytokine cocktail containing Flt3 ligand. The manipulated cells were reinfused into the patient after a 4-day in vitro transduction, and the regimen was repeated twice for each patient. As in the p47^{phox} study, oxidase-positive neutrophils were detected 3–4 weeks after each infusion, at a slightly higher frequency (0.06–0.2%). Nonetheless, the number of circulating gene-corrected neutrophils declined over time. Both of these studies suggest that marrow conditioning prior to infusion of transduced cells will be important for a higher level engraftment of corrected stem/progenitor cells, in addition to the development of more efficient gene transfer techniques for the transduction of human stem cells.

Conclusion

CGD is a promising candidate disease for the development of gene therapy targeted at marrow-derived cells. Based on clinical observations of female X-CGD carriers and on studies in murine CGD models, correction of respiratory burst oxidase activity in even as few as 10–20% of phagocytes is likely to be of some clinical benefit. Although even this degree of correction would be difficult to obtain with current approaches, improvements in transplant conditioning and vector design may permit the achievement of clinically relevant levels of corrected neutrophils for human CGD patients. The in vivo selection of transduced hematopoietic cells is another potential approach to increase the percentage of gene-corrected blood cells, as suggested by murine studies utilizing linked drug resistance markers such as the multidrug resistance (MDR) protein or dihydrofolate reductase [64–66]. In addition to the above findings which are specific for

CGD, insights into basic hematopoiesis and strategies for gene transfer into hematopoietic stem cells gained from these CGD studies will be applicable to many other aspects of stem cell transplantation and gene therapy of stem cell disorders.

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Hematoprotection by Transfer of Drug-Resistance Genes

Michael Flasshove Thomas Moritz Walter Bardenheuer Siegfried Seeber

Department of Internal Medicine (Cancer Research), West German Cancer Center, University of Essen Medical School, Essen, Germany

Key Words
Gene therapy · Hematopoietic stem cells · Drug resistance · Retroviral vector

Abstract
Myelosuppression represents a major side effect of cytotoxic anti-cancer agents. Infection due to granulocytopenia and the risk of bleeding due to thrombocytopenia compromise the potential of curative and palliative chemotherapy. Considering the many chemotherapeutic agents for which drug resistance genes have been described, and the recent improvements in vector and transduction technology, it seems conceivable that drug resistance gene transfer into a patient's autologous hematopoietic stem or progenitor cells will be able to reduce or abolish chemotherapy-induced myelosuppression.

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Introduction

Treatment of malignant tumors usually combines several modalities such as irradiation, surgery, and systemic chemotherapy. For hematological malignancies, lymphoma and leukemia, chemotherapy represents the mainstay of treatment. Solid tumors often require chemotherapy with curative or palliative intention, especially in more advanced stages. For these malignancies, dose-intensified or high-dose chemotherapy as a therapeutic option has been under investigation for the last decade. Results from randomized studies suggest that patients with advanced multiple myeloma [1] and relapsed high-grade non-Hodgkin's lymphoma [2] should be offered high-dose chemotherapy. Moreover, patients with advanced or refractory/relapsed germ cell tumors seem to benefit from high-dose chemotherapy [3, 4]. For several other entities, such as breast cancer, the role of high-dose chemotherapy has yet to be defined [5].

Since chemotherapeutic drugs do not exclusively affect malignant cells, toxicity to normal tissue resulting in debilitating or life-threatening loss of organ function limits the dosage of these substances. Although the dose-limiting toxicity varies from drug to drug and can involve nearly all organs, myelotoxicity entailing neutropenia, thrombocytopenia, and anemia usually represents the most dominant clinical problem. Myelosuppression may even prevent the application of adequate doses of chemotherapy, leading to lower remission rates and shorter survival in treatment protocols with curative or palliative intention. A transplantable population of hematopoietic progenitor cells, rendered resistant to chemotherapeutic drugs ex vivo, might help to circumvent this problem.

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Fax + 41 61 306 12 34
E-Mail karger@karger.ch
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Priv.-Doz. Dr. Michael Flasshove
Department of Internal Medicine (Cancer Research)
West German Cancer Center, University of Essen Medical School
Hufelandstrasse 55, DE–45122 Essen (Germany)
Tel. +49 201 723 3116, Fax +49 201 723 2178, E-Mail michael.flasshove@uni-essen.de

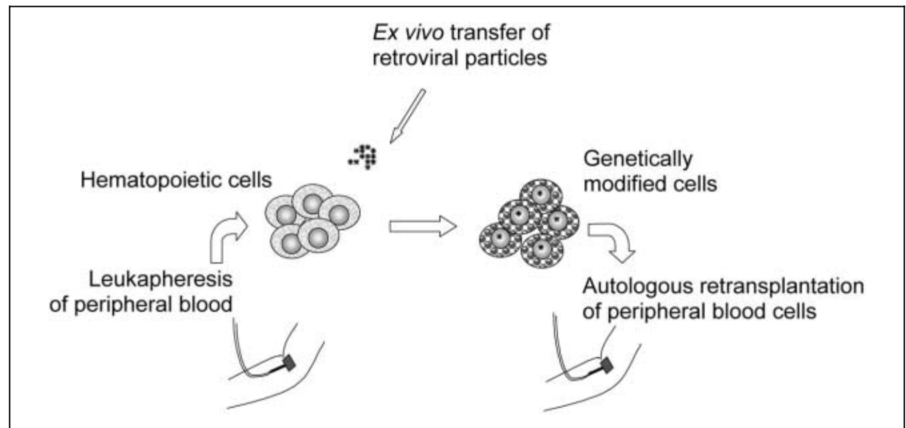

Fig. 1. Principle of hematopoietic cell gene therapy. For details see text.

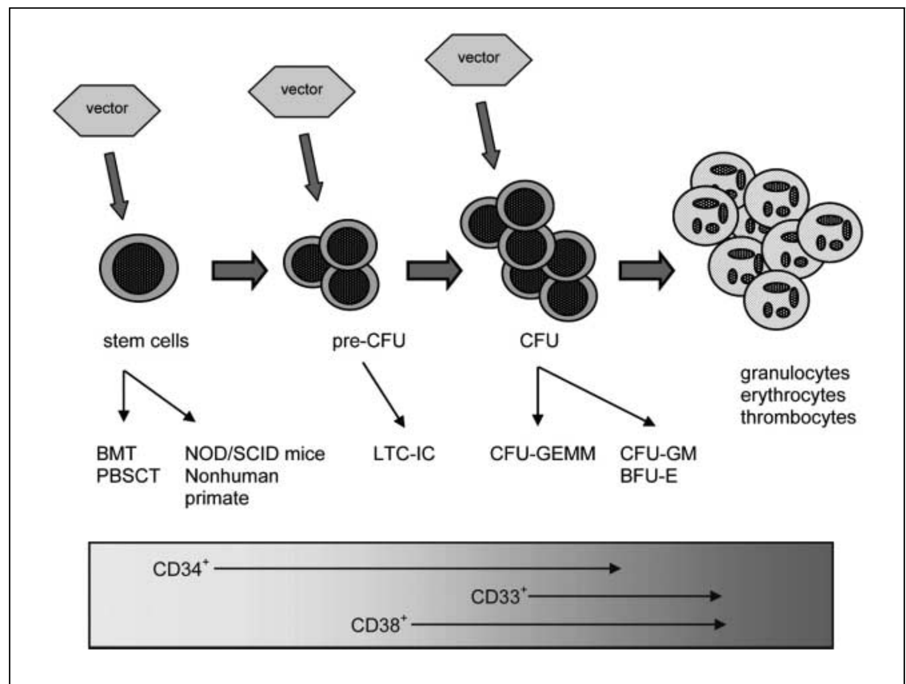

Fig. 2. The human hematopoietic compartment. BMT = Bone marrow transplantation; PBSCT = peripheral blood stem cell transplantation; NOD/SCID = nonobese diabetic/severe combined immunodeficient; LTC-IC = long-term culture-initiating cells; CFU = colony-forming unit; CFU-GEMM = colony-forming unit-granulocyte erythroid megakaryocyte macrophage; CFU-GM = colony-forming unit-granulocyte macrophage; BFU-E = burst-forming unit-erythroid.

As many prerequisites for such a concept are available right now, future clinical trials should be able to prove the feasibility of this approach. Figure 1 schematically depicts the clinical concept that is based upon the (i) mobilization of bone marrow-residing hematopoietic progenitor and stem cells into the peripheral blood of patients after application of chemotherapy and hematopoietic growth factors and the collection of these cells via standard leukapheresis procedures; (ii) ex vivo manipulation of these hematopoietic cells, and finally (iii) autologous retransplantation followed by application of dose-intensive and myelosuppressive chemotherapy. It is conceivable that such an approach can target different subpopulations of the hematopoietic compartment. As shown in figure 2, human hematopoiesis is characterized by a hierarchical order from pluripotent stem cells to determined progenitor cells and, finally, the most mature peripheral blood cells.

A major goal of hematopoietic cell gene therapy has always been the modification of true long-term repopulating stem cells, and this is probably a prerequisite for the treatment of monogenetic disorders. Therefore, the successful transfer of the gene correcting X-linked severe combined immunodeficiency syndrome into pluripotent hematopoietic cells has been a milestone for the clinical development of this technology [6]. For the purpose of hematoprotection from cytotoxic drugs two approaches

Table 1. Chemotherapy resistance genes

Gene	Resistance[1]	Mechanism	References[1]
Multidrug-resistance gene 1	Anthracyclines, vinca alkaloids, taxanes, etoposide	Transmembrane drug efflux pump	58, 60 62–65
Multidrug-resistance-associated protein	Anthracyclines	Transmembrane drug efflux pump	87
hENT2 nucleoside transporter	Trimetrexate, tomudex and NBMPR[2]	NBMPR insensitive nucleoside transporter	53
Dihydrofolate reductase	Methotrexate, trimetrexate	Mutant form is not inhibited by drug	8, 25, 32, 40
Cytidine deaminase	Cytosine arabinoside, gemcitabine	Intracellular detoxification	35, 97, 98
Aldehyde dehydrogenase	Cyclophosphamide	Intracellular detoxification	36, 133
Glutathione S-transferase	Cyclophosphamide, anthracyclines	Intracellular detoxification	138, 139
Thymidylate synthase	5-Fluorouracil, tomudex	Mutant form is not inhibited by drug, expression of humanized *E. coli* enzyme	54–56
γ-Glutamylcysteine synthetase and MRP1[3]	Anthracyclines, melphalan	Intracellular detoxification and transmembrane drug efflux pump	88
O^6-Methylguanine DNA methyl-transferase	Chloroethylnitrosoureas	Repairs O^6 adducts of the DNA	103–105
apn1	Bleomycin	Repairs bleomycin-induced DNA damage	121
Formamidopyrimidine DNA glycosylase or oxoguanine DNA glycosylase	Thiotepa	Repairs Fapy lesions of DNA	122

NBMPR = Nitrobenzylmercaptopurine riboside; apn1 = major yeast AP (apurinic/apyrimidinic) endonuclease; MRP1 = multidrug-resistance-associated protein-1.

1 Chemotherapeutic drugs and references listed are confined to data for hematopoietic cells.

2 Either trimetrexate or tomudex were given in combination with NBMPR.

3 Experiments were performed with a vector encoding both cDNAs.

may be successful. On the one hand, the transfer of chemotherapy resistance genes into true stem cells would guarantee long-lived expression in all progeny. However, there are still technical problems associated with this approach. On the other hand, expression of these genes in a more determined progenitor cell population, such as colony-forming units (CFU) or pre-CFU (fig. 2), which seems to be feasible with current technology, should also result in a sufficient number of transplantable drug-resistant progenitor cells. These cells may supply enough differentiated peripheral blood cells to protect the hematopoietic system during chemotherapy.

The transfer of exogenous genetic material into primary human hematopoietic cells can be achieved with different viral vector systems. After the first stable transgene expression in murine and human hematopoietic cells was reported in the mid 1980s [7–12], most preclinical and clinical work has been done with retroviral vectors. This technology has recently been reviewed [13]. Several generations of safety-modified vectors derived from (onco-) retroviruses have been published, the most successful ones using sequences from Moloney murine leukemia virus (MMLV) [14, 15] or spleen focus-forming virus (SFFV) [16]. More recently, promising constructs on the basis of lentiviruses such as HIV have been reported and might help to overcome some of the problems unsolved by conventional retroviral vectors, e.g. the low transduction efficiency for quiescent stem cells [17, 18].

So far, numerous genes have been identified, which are capable of conferring resistance to chemotherapeutic drugs after transfer into human or murine cells. Table 1 comprises only those genes, whose expression has been shown to result in increased resistance in cells of the hematopoietic compartment. They can roughly be divided into 3 subgroups according to the mechanism that is responsible for the drug resistance: (i) genes encoding

membrane-bound proteins functioning as efflux pumps for cytotoxic drugs, such as the multidrug-resistance gene 1 (MDR1); (ii) genes encoding cytoplasmatic proteins that are involved in the intracellular activity or metabolism of cytotoxic drugs, such as mutant forms of dihydrofolate reductase (DHFR) or cytidine deaminase (CDD), and (iii) genes encoding nuclear proteins with the ability to repair the DNA damage caused by cytotoxic drugs, such as O^6-methylguanine DNA methyltransferase (MGMT). Some of these genes act by (over-)expression of the wild-type protein in the respective target cells, such as MDR1 or CDD. Others encode mutant proteins not affected by the respective cytotoxic drug, such as DHFR. Certainly, there are more candidate genes suitable for hematoprotection than those listed in table 1, but this review will focus on the genes already established to confer resistance to human or murine hematopoietic cells.

Dihydrofolate Reductase

DHFR is a critical enzyme of the intracellular folate metabolism catalyzing the reduction of dihydrofolates to tetrahydrofolates, which are essential for thymidilate and purine synthesis [19]. The basic principle of chemotherapy resistance conferred by the DHFR gene consists in the use of mutant forms ($DHFR^{mut}$), which still exhibit their enzymatic function but will be inhibited by methotrexate (MTX) or related compounds such as trimetrexate (TMTX) to a much lesser degree than the wild-type enzyme [20–22]. The most widely used single-point mutations have been the leucine → arginine substitution at codon 22 ($DHFR^{Arg22}$) and the phenylalanine → serine substitution at codon 31 ($DHFR^{Ser31}$) of the DHFR cDNA. More recently, double-point mutations have been cloned which show a much higher ability to confer MTX resistance [23, 24]. $DHFR^{mut}$ has been the first drug-resistance gene used for retrovirally mediated gene transfer into murine hematopoietic cells [8, 25], and increased MTX resistance after retroviral expression of $DHFR^{mut}$ has been reported for committed progenitor cells of murine, canine, and human origin [8, 25–27]. Transfer of the $DHFR^{Ser31}$ into transplantable murine progenitor cells resulted in protection from MTX toxicity in a mouse in vivo model [28, 29]. Moreover, transplantation of $DHFR^{Ser31}$-transduced murine bone marrow cells into mice bearing a pre-established mammary tumor allowed a curative MTX treatment, which was lethal to the control mice [30]. The same retroviral construct conferred MTX resistance to primary human hematopoietic cells isolated from peripheral blood and umbilical cord blood [31, 32].

In contrast to other cytotoxic drugs, myelosuppression is not the critical toxicity following antifolate single-agent therapy. Nevertheless, MTX may significantly contribute to the myelotoxicity of combination chemotherapy regimens and the myeloprotective effect of the combined expression of $DHFR^{mut}$ with other drug-resistance genes, in particular MDR1, CDD, and aldehyde dehydrogenase (ALDH) is presently investigated [33–36]. Moreover, $DHFR^{mut}$ gene transfer may be utilized for allogeneic stem cell transplantation. In this situation, antifolates used for prophylaxis of graft-versus-host disease, are particularly hematotoxic and frequently delay hematopoietic reconstitution [37].

Transfer of $DHFR^{mut}$ may not only be useful in MTX-containing chemotherapy regimens, but also to select for successfully transduced cells, which coexpress a second gene of interest. Especially, gene transfer for the correction of monogenetic hematological diseases may require such an approach to achieve therapeutic efficiency. The application of MTX or TMTX has been demonstrated to allow enrichment of $DHFR^{mut}$-transduced cells in vitro or in vivo in a mouse transplant model [38–41]. Successful in vitro selection has also been described for hematopoietic cells of human origin [31, 32, 42, 43]. One has to keep in mind though, that in vitro and in vivo selection procedures up to now require the inhibition of the cellular thymidine uptake from serum as a potential salvage mechanism from MTX-induced toxicity. This can be achieved in vitro by using dialyzed serum [25], serum-free media [41], or pretreatment of the serum with thymidine phosphorylase [27], but these techniques cannot be applied in vivo. So far, it is unknown how much the thymidine concentration in human plasma, which is in the order of 2×10^{-7} mol/l [44] and, therefore, lower than the concentration necessary for complete inhibition of MTX toxicity [45], could influence the in vivo drug resistance and selectibility of $DHFR^{mut}$-transduced human hematopoietic cells. The simultaneous application of nucleoside transport inhibitors, such as dipyridamole or NBMPR, may circumvent the salvage thymidine uptake from serum in vivo, but this approach has only been used in the murine system thus far [39, 40, 46]. Another question yet to be answered relates to the subpopulation of hematopoietic cells which is sensitive to MTX. Here, at least for the murine hematopoietic compartment, conflicting data have been reported. Blau et al. [47] described MTX toxicity only at the level of more differentiated nonclonogenic progenitor cells, while Allay et al. [46] demonstrated suc-

cessful in vivo selection of DHFR^{mut}-expressing repopulating stem cells when administering MTX in combination with NBMPR in mice.

Thus, several issues have to be addressed before clinical studies with DHFR^{mut} will be feasible. Whereas in the murine system convincing data for the protection of hematopoietic cells from MTX toxicity have been reported by several groups over the past years including in vivo transplantation studies [25, 29, 30, 46, 48], data on human hematopoietic cells have been limited to in vitro experiments and the level of transferred MTX resistance was only modest [31, 32, 36, 41, 43, 49]. The degree of MTX resistance could be increased by using double-point instead of single-point mutated DHFR cDNA, an observation initially described for fibroblasts [23] and murine bone marrow cells [24]. More recently, these data have been confirmed for human hematopoietic progenitor cells from peripheral blood, bone marrow, and umbilical cord blood, and protection against concentrations of up to 10^{-5} mol/l MTX as well as successful selection could be achieved [42, 50]. These improved results reflect the use of double-point mutated DHFR in combination with more effective retroviral backbones and are encouraging with respect to the performance of future clinical trials. Moreover, coexpression of DHFR^{mut} together with enzymes involved in thymidine or purine salvage pathways such as herpes simplex virus thymidine kinase or xanthine-guanine phosphoribosyltransferase may even potentiate the level of MTX resistance conferred to successfully targeted hematopoietic cells [51, 52].

Nucleoside Transporter

Another strategy to transfer drug resistance, closely related to the DHFR metabolism and the nucleotide salvage pathways, has recently been published by Patel et al. [53]. Several membrane-spanning nucleoside transporter molecules are known to salvage extracellular nucleosides, thereby circumventing antifolate-induced toxicity. In humans, high expression of the *es* nucleoside transporter has been found in normal tissue as well as in tumor cells. In contrast, the *ei* nucleoside transporter seems to be present only at low levels in human cells. Since NBMPR is a potent inhibitor of the *es* but not of the *ei* nucleoside transporter, retroviral transduction of hematopoietic cells with the *ei* gene followed by combined treatment with an antifolate and NBMPR (potentially highly cytotoxic to only *es* expressing cells) resulted in significant hematoprotection in vitro and in vivo in a mouse model [53]. Given these data, it might also be interesting to combine the transfer of the *ei* transporter with the DHFR^{mut}.

Thymidylate Synthase

A similar approach as chosen for imparting MTX resistance on hematopoietic cells (the use of a mutant protein) has also been applied to generate resistance to drugs exhibiting their cytotoxic effect through inhibition of the enzyme thymidylate synthase (TS) [19]. Several mutants of TS have been described, whose transfer increases cellular resistance to 5-fluoro-2-deoxyuridine, raltitrexed, thymitaq, or BW1843U89 and may be useful for hematoprotection [54, 55]. Alternatively, *Escherichia coli* TS, which seems to be more active than human TS, with codons optimized for expression in mammalian cells has been used for transduction [56]. However, data reported so far for TS gene transfer to hematopoietic cells have been limited to in vitro experiments.

Multidrug-Resistance Gene 1

The MDR1 gene encodes the P-glycoprotein, a drug efflux pump localized in the cell membrane and removing cytotoxic drugs such as anthracyclines, vinca alkaloids, and taxanes from the cytoplasm. Therefore, elevated expression of MDR1 in cancer cells is correlated with increased drug resistance. The potential therapeutic application of this gene is based on the observation that transduction of cell lines with a retroviral vector encoding MDR1 resulted in a drug-resistant phenotype [57]. Successful transduction with MDR1-encoding vectors has also been demonstrated in hematopoietic cells leading to increased resistance against cytotoxic drugs [58–65]. Moreover, transfer of MDR1 into transplantable murine hematopoietic cells allowed successful in vivo selection of the transplanted cells in the recipient mice [58]. However, splice variants of the MDR1 cDNA reducing the amount of functional protein expressed from several retroviral vectors have been identified as a potential problem [66, 67], and improved expression of MDR1 has been observed after gene transfer of splice-corrected MDR1 cDNA [34, 68]. Retroviral vectors based on a spleen focus-forming virus/murine embryonic stem cell virus hybrid seem to improve the expression of MDR1 in human hematopoietic cells [16, 60, 68]. Use of these vectors resulted in successful gene transfer of human peripheral blood progenitor cells capable of long-term repopula-

tion after transplantation into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice [68, 69].

As discussed for the use of $DHFR^{mut}$, the MDR1 cDNA may potentially serve as a selection marker that allows in vitro or in vivo selection of successfully transduced cells. This has been shown for murine as well as human hematopoietic cells [58, 62, 70]. Thus far, it is unclear whether selection with MDR1-associated cytotoxic drugs will ultimately result in a selection of true human hematopoietic stem cells or will be confined to hematopoietic progenitor cells, because a high endogenous MDR1 expression has been described for undifferentiated hematopoietic progenitor/stem cell populations, including SCID-repopulating cells [71, 72]. Moreover, in contrast to $DHFR^{mut}$ gene transfer in combination with MTX treatment, the pronounced nonhematopoietic side effects of MDR1-associated drugs, such as cardio- and neurotoxicity, have to be taken into consideration for such a clinical approach.

Clinical Trials

Several clinical trials using MDR1-encoding vectors have been reported. In all studies autologous peripheral blood or bone marrow cells were retransplanted after ex vivo retroviral transduction into patients with breast cancer, ovarian cancer, lymphoma, or germ cell tumors [73–79]. Some clinical trials used a Harvey murine sarcoma virus (HaMSV)-based vector [74, 76, 79], others used MMLV-based constructs [73, 75, 77, 78]. In general, these trials have met with little success. Nevertheless, one study reported a correlation between the relative number of MDR1-containing granulocytes and the granulocyte nadir after paclitaxel treatment despite a low level of engraftment of MDR1-positive cells [73]. Another trial observed the expansion of MDR1-transduced hematopoietic cells (estimated by the presence of proviral DNA in peripheral blood granulocytes) after treatment with MDR1-associated drugs [77]. More encouraging results have recently been reported by Abonour et al. [79] from a clinical study in patients with germ cell tumor. An optimized transduction protocol making use of the matrix molecule fibronectin enabled increased transfer efficiency into undifferentiated hematopoietic cells, and resulted in persistent marking of hematopoietic cells at considerable levels for more than a year after transplantation [79]. The level of expression was obviously limited by a high rate of aberrant splicing of the MDR1 gene, as already known for the HaMSV vector used in this study [66].

Thus, clinical trials so far have only shown the feasibility of the ex vivo manipulation required for the genetic modification of peripheral blood stem cells. The feasibility and safety of the transplantation of MDR1-transduced cells will only be proven when, in fact, the transfer efficiency and the level of expression of the transgene MDR1 is high enough to protect against chemotherapy-induced myelotoxicity. Considering safety issues one has to keep in mind that vectors expressing MDR1 at a high level have caused a so-called ‘myeloproliferative syndrome’ in mice after transplantation of transduced and ex vivo expanded hematopoietic cells [80]. Another observation is the increased number of ‘side population’ stem cells in the murine hematopoietic compartment after MDR1 gene transfer into murine bone marrow cells [81], indicating a profound effect on the hematopoietic stem cell compartment at least in mice. These observations may be limited to specific types of retroviral vectors, such as HaMSV-based vectors, but the issue certainly needs further evaluation with respect to future clinical trials.

Hopefully, new retroviral vectors [16], reduced aberrant splicing by correction of internal splice sites [34], and optimized clinical-scale transduction protocols [82] will enable the performance of meaningful clinical studies of MDR1 gene transfer to answer the question of clinical benefit for the patient in the near future.

Multidrug-Resistance-Associated Protein

The multidrug-resistance-associated protein (MRP) is a member of the ATP-binding cassette transporter superfamily and a transmembrane efflux transporter similar to P-glycoprotein/MDR1 [83]. The detoxifying function of MRP depends on the presence of glutathione [84]. The phenotype of MRP-expressing cells is slightly different from MDR1-positive cells and comprises resistance to anthracyclines, vinca alkaloids and epipodophyllotoxins, but not taxanes [85]. Increased resistance against doxorubicin, vincristine, and etoposide has been reported for retrovirally transduced NIH 3T3 fibroblasts [86]. Successful transduction of long-term repopulating cells in a murine transplantation model mitigated the doxorubicin-induced leukopenia of MRP-positive mice [87]. Confirming evidence for the hematoprotective effect of MRP gene transfer came from more recent studies which revealed efficient transduction of murine hematopoietic colony-forming cells with a vector coexpressing γ-glutamylcysteine synthetase (γ-GCS) and MRP resulting in resistance to etoposide and melphalan [88].

Cytidine Deaminase

The potential role of the CDD as a drug-resistance gene was derived from the metabolism of the pyrimidine analog cytosine arabinoside (ara-C). CDD deaminates cytosine nucleosides and their analogs, such as ara-C, preventing the accumulation of intracellular ara-CTP, the active metabolite of ara-C [89]. Ara-C together with anthracyclines is the most active drug for the treatment of acute myeloid leukemia and is effective in combination chemotherapy for acute lymphocytic leukemia and non-Hodgkin's lymphoma. Low-dose as well as high-dose ara-C schedules result in severe and life-threatening myelosuppression. Increased resistance to ara-C has initially been observed in human myeloblasts and cell lines overexpressing CDD [90, 91]. With the cloning of a functional cDNA of the CDD [92, 93] the analysis of targeted (over)-expression in specific cell types became possible. Consequently, stable transfection of the CDD in murine fibroblast cell lines was demonstrated to result in increased resistance to ara-C [94, 95]. Retroviral transduction of the CDD cDNA was also applied to increase ara-C resistance in hematopoietic cell lines [96]. So far, 3 groups have reported increased ara-C resistance after retrovirally mediated transduction of primary murine bone marrow cells [35, 97, 98]. While 2 investigators found only modest levels of ara-C resistance [35, 97], the degree of ara-C resistance observed by Momparler et al. [98] was much more pronounced and was confirmed later by the same group in an in vivo mouse transplant model [99]. More recently, Beauséjour et al. [100] observed that transfer of CDD into murine bone marrow cells would allow enrichment of successfully transduced cells.

Thus far, data for the successful transduction of primary human hematopoietic cells are missing, although human CD34+ cells seem to have low CDD enzymatic activity [95], rendering them an excellent target cell population for this approach. In this context, the observation of Gran et al. [101] may be of importance. They reported growth inhibition of murine and human granulocyte-macrophage colony-forming cells when cultured in the presence of recombinant human CDD protein [101]. Thus, in clonogenic progenitor cells overexpressing CDD a potential inhibitory effect might compensate for the anticipated growth advantage in the presence of ara-C. One has to keep in mind, though, that the effect observed by Gran et al. [101] was dependent on the presence of 10^{-4} mol/l thymidine, a concentration much higher than the 2×10^{-7} mol/l thymidine usually present in human plasma [44].

Data on primary human cells have to be awaited before the potential clinical benefit of CDD gene transfer can be defined. Moreover, the clinical scenario involving autologous transplantation of genetically modified cells, as envisioned for other drug-resistance genes, may not be applicable to CDD, at least for ara-C-containing chemotherapy protocols. Leukemias and lymphomas, usually treated with ara-C, are at high risk for minimal residual disease in bone marrow or peripheral blood. Thus, accidental transduction of malignant cells cannot be ruled out and may jeopardize a safe autologous retransplantation of transduced cells into the patient. However, Eliopoulos et al. [102] reported increased drug resistance after CDD gene transfer not only for ara-C, but also for other cytosine nucleoside analogs, such as 5-aza-2′-deoxycytidine and 2′,2′-difluorodeoxycytidine (dFdC; gemcitabine). Especially dFdC has become an important first-line or second-line chemotherapeutic drug for many malignancies including lung cancer and pancreatic cancer, with myelotoxicity as a side effect often requiring dose reduction in palliative treatment concepts.

O^6-Methylguanine DNA Methyltransferase

MGMT belongs to the group of DNA repair proteins and has been evaluated as a candidate for a clinical approach of transferring drug resistance to hematopoietic cells [103–105]. While other DNA repair pathways involve the concerted action of multiple proteins, repair by MGMT is a one-step mechanism in which the damaging alkyl group at the O^6 position of guanine is transferred to a cysteine residue within the acceptor pocket of the MGMT protein [106]. Bone marrow cells including the CD34+ subpopulation show very low MGMT activity as compared with other human tissues [107, 108], which might explain the profound hematotoxicity of O^6-alkylating agents. The feasibility of hematoprotection by MGMT overexpression has first been shown for wild-type MGMT [104, 105, 109, 110], but the increase in chemotherapy resistance was only moderate and selection of transduced cells in in vivo transplantation models was not satisfying [103, 111]. A number of benzylguanine (BG)-resistant forms of MGMT have been evaluated for their potential to protect hematopoietic cells from the toxicity of combined BG/O^6-alkylating agent application. BG is a guanine analog, which binds to the catalytic center of MGMT and causes irreversible inactivation of the protein [112]. It has been used in clinical phase-I studies to deplete MGMT-overexpressing tumors of O^6-alkyl repair activi-

ty, thereby increasing the cytotoxic effect of O^6-alkylating agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) [113]. BG-resistant forms of MGMT include the bacterial ada gene, as well as a number of single amino acid mutations of the human MGMT gene, such as P140A, G156A, or P140K. Retrovirally mediated expression of MGMT mutants has been used to protect primary murine and human hematopoietic cells in vitro [114, 115], and murine bone marrow in an in vivo transplantation model [116, 117]. In particular, use of the P140K mutant, which in comparison to wild-type MGMT is characterized by a more than 1,000-fold increased resistance to BG, has been promising. The transfer of this mutant allowed selection of transduced murine bone marrow cells in vivo in primary as well as secondary recipients of transduced bone marrow, indicating that selection occurred even at the level of stem cells [117, 118].

One major representative of O^6-alkylating agents is BCNU, which is effective in the treatment of brain tumors. Moreover, it has therapeutic activity in small-cell lung cancer and lymphoma. Since severe pulmonary fibrosis has been described as a dose-limiting side effect of BCNU therapy, in particular at high doses [119], other O^6-alkylating agents, such as the chloroethylnitrosourea-type compounds, CCNU and ACNU, or the hydrazine and triazene derivatives, procarbazine, temozolomide, and dacarbazine, may be even more promising for this clinical approach. In addition to the clinical applications already mentioned, triazene-type drugs also show cytotoxic activity in malignant melanoma and soft tissue sarcoma. After retroviral transduction of hematopoietic progenitor cells with wild-type MGMT, drug resistance against several of these drugs, such as CCNU, BCNU, ACNU and, at a lower level, also against temozolomide has been demonstrated [120]. Thus, transfer of MGMT might ultimately be used to increase resistance against a variety of different cytotoxic compounds useful for the curative or palliative treatment of many types of cancer.

Other Chemotherapy-Resistance Genes of the DNA Repair Systems

In addition to the MGMT, several other candidate chemoresistance genes involved in DNA repair have been described, which need further exploration in primary hematopoietic cells (table 1). Apn1 is the major yeast apurinic/apyrimidinic (AP) endonuclease in the DNA base excision repair pathway, and retrovirally mediated apn1 expression seems to protect murine bone marrow in vitro and in vivo from bleomycin-induced toxicity [121]. Other proteins involved in base excision repair – the bacterial formamidopyrimidine DNA glycosylase (fpg) and its human functional equivalent oxoguanine DNA glycosylase (OGG1) – have been shown to confer resistance to thiotepa after retroviral gene transfer into murine bone marrow cells in vitro. Moreover, fpg gene transfer resulted in increased resistance to thiotepa and allowed successful selection of transduced hematopoietic cells in a murine in vivo model [122].

Aldehyde Dehydrogenase

The class-1 and class-3 ALDHs have been linked to the inactivation of cyclophosphamide. Cyclophosphamide is metabolized in the liver by cytochrome P-450 enzymes to its active compounds, the first being 4-hydroxycyclophosphamide and its open-ring tautomer, aldophosphamide. Mafosfamide and 4-hydroperoxycyclophosphamide are stabilized precursors converted to the active compound 4-hydroxycyclophosphamide in aqueous solution and, therefore, suitable for in vitro application [123, 124]. The human ALDH detoxifies the active metabolites of these drugs by oxidation to the inactive carboxyphosphamide [124], and intracellular ALDH activity seems to determine cyclophosphamide resistance of specific cell types [125]. Human hematopoietic progenitor cells show a high intracellular content of ALDH [126], which has been used for the isolation of primitive progenitor cell subpopulations via flow cytometry [127] and at the same time, may provide a possible explanation for the stem cell-sparing myelosuppression of cyclophosphamide in vitro and in vivo [128]. Transfection with either isoenzyme, cytosolic ALDH-1 or cytosolic/microsomal ALDH-3 cDNA, resulted in increased resistance in cell line experiments, with a higher level of resistance observed for the use of ALDH-1 [129, 130].

Data on the transfer of ALDH into hematopoietic cells are very limited. Increased resistance was reported for K562 leukemia cells after retroviral transduction and additional selection with 4-hydroperoxycyclophosphamide [131, 132]. Magni et al. [133] reported a 4- to 10-fold increase in resistance against mafosfamide for human clonogenic progenitor cells from mobilized peripheral blood, whereas data from Bunting et al. [134] suggested a low protein expression due to a short half-life of the ALDH-1 mRNA expressed from various retroviral vectors. More recently, successful protection of NIH 3T3 fibroblasts, CD34-enriched human peripheral blood progenitor cells, and transplantable murine bone marrow

cells from 4-hydroperoxycyclophosphamide toxicity could be obtained using a retroviral vector encoding ALDH-1 and $DHFR^{mut}$ [36]. Additional data, especially on human primary cells, have to be awaited before the role of ALDH as a drug-resistance gene in hematoprotective strategies can be definitely determined. This seems to be of great interest because even today cyclophosphamide is one of the most commonly used cytostatic drugs. Since cyclophosphamide does not affect very primitive hematopoietic progenitor cells, ALDH will probably not be useful in a stem cell selection strategy.

Glutathione S-Transferase and γ-Glutamylcysteine Synthetase

A well-known mechanism of drug resistance to alkylating agents is linked to the intracellular level of glutathione (GSH), which binds to nearly all alkylating agents, including melphalan, cyclophosphamide, etc. [124]. The glutathione S-transferase (GST) isoenzymes catalyze the formation of GSH conjugates with the respective alkylating drug, while the γ-glutamylcysteine synthetase (γ-GCS) represents the rate-limiting step of the GSH synthesis. Thus, overexpression of both enzymes should increase the formation of GSH conjugates and, consequently, also lead to an increase in resistance to the respective drug. Retroviral transduction of rat GST-Yc has been reported to confer resistance to chlorambucil in NIH 3T3 mouse fibroblasts [135]. More recently, coexpression of rat GST-A3 (previously designated as rat $GST\text{-}Yc_1$) in combination with CDD resulted in a several-fold increased resistance of NIH 3T3 fibroblasts against melphalan, chlorambucil, and ara-C [136]. As observed for other drug resistance genes, such as MGMT and CDD, the expression of GST, at least of GST-α, seems to be low in human CD34+ cells [137]. A significant resistance of CFU-GM against adriamycin and 4-hydroxycyclophosphamide could be observed after retrovirally mediated introduction of human GST-π [138]. The same group reported successful transfer of GST-π into murine hematopoietic cells in a transplantation model allowing for high-dose cyclophosphamide treatment of primary recipients and resulting in GST-π expression in CFU-GM generated from secondary recipients as an indication for successful gene transfer into long-term repopulating cells [139]. More recently, an SFFV-derived retroviral vector was used to transduce murine hematopoietic cells with both the MRP1 and the γ-GCS. Significant resistance of CFU against etoposide and melphalan could be observed [88].

Combination of Drug-Resistance Genes

For most clinical approaches the combined expression of several drug-resistance genes or the expression of a drug-resistance gene together with a nonselectable therapeutic gene seems to be a desirable goal. As a matter of fact, several investigators have reported the feasibility of coexpressing two genes from the same retroviral vector using additional internal promoters, fusion genes, or internal ribosomal entry sites. Functional resistance to at least two different cytotoxic drugs could be observed, e.g. MDR1 and $DHFR^{mut}$ [34], ALDH and $DHFR^{mut}$ [36], CDD and GST [136], MDR1 and MGMT [140], TS and $DHFR^{mut}$ [141], and MRP1 and γ-GCS [88].

Safety Aspects

The safety of drug-resistance gene transfer in clinical approaches has been a permanent matter of discussion. Several issues have to be taken into consideration: (i) an accidental infection of the patient with helper virus, as was reported in early nonhuman primate experiments [142], seems to be unlikely, because the use of this technology for hematoprotection will be strictly confined to an ex vivo gene transfer allowing for all kinds of analyses before transduced cells would be retransplanted to the patient; (ii) adverse effects on the target cells through overexpression of the gene of interest, such as the recently observed 'myeloproliferative syndrome' after transplantation of MDR1-transduced cells into mice [80] can neither be ruled out from in vitro data nor from the clinical trials performed thus far, since most clinical trials have suffered from low gene transfer efficiency and low expression of the transgene; (iii) disruption of an important gene by the randomly integrating retroviral vector (insertional mutagenesis) has been more a theoretical consideration for many years, but more recently, this has been observed in a mouse transplant model [143] as well as in children treated for X-linked severe combined immunodeficiency syndrome [6, 144], resulting in a leukemia-type disease in both cases [143, 144], and (iv) finally, the accidental transduction of a contaminating malignant cell is a major concern for the transfer of chemotherapy-resistance genes [145], requiring careful analysis of the transduced cells before transplantation. Improved purging techniques will have to be combined with sensitive assays for minimal residual disease to guarantee autotransplants free of contaminating tumor cells in clinical studies.

Conclusion

Since the first successful gene transfer into hematopoietic cells with safety-modified retroviral vectors was reported, many efforts have been made to improve and optimize this system in order to finally reach clinical applicability. During this time, approximately 15 years now, quite a few clinical trials were started and, in most cases, failed. With the recent improvements in vector and transduction technology on the one hand, and the widespread and routine use of autologous transplantation of peripheral blood progenitor cells on the other hand, it has now again become an intriguing idea to reduce or abolish myelosuppression as one of the major side effects of chemotherapy. In this context, hematoprotection may be achieved through efficient transduction of more differentiated progenitor cells, as well as transduction of true long-term repopulating stem cells. Although myelosuppression is not the sole dose-limiting toxicity of most of these drugs, infection due to granulocytopenia and the risk of bleeding due to thrombocytopenia compromise the potential of curative and palliative chemotherapy. Considering the various chemotherapeutic drugs for which drug-resistance genes have been described, it seems conceivable that drug-resistance gene transfer could circumvent myelotoxicity for most, if not all, chemotherapeutic regimens.

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Gene Therapy Targeting Hematopoietic Cells: Better Not Leave It to Chance

Christopher Baum[a,b] Christof von Kalle[b]

[a]Department of Hematology and Oncology, Hannover Medical School, Hannover, Germany;
[b]Division of Experimental Hematology, Cincinnati Children's Hospital, Cincinnati, Ohio, USA

Key Words
Gene therapy · Retroviral vector · Mutagenesis · Transformation

Abstract
Gene therapy targeting hematopoietic cells has arrived at a new stage of potency. While the potential for curing inherited disorders of the immune system has been demonstrated in clinical trials, we were also confronted with the first serious adverse events related to random insertion of foreign DNA into cellular chromosomes. As it is likely that the manifestation of severe side effects results from a multifactorial process, it will be of crucial importance to define the significance of the individual risk factors involved. The future of the field will depend on our ability to define risk classifications of clinical approaches, to continuously improve transgene technologies, and to introduce new concepts for targeted selection of transgenic cells. Interestingly, correction of genetic disorders by homologous gene repair in defined stem cell clones is on the horizon, but far from being available for clinical use.

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Gene therapy aims to cure inborn or acquired genetic disorders by corrective gene transfer into somatic cells. In the past year (2002), several key observations were made illustrating the whole spectrum of promises and pitfalls encountered with this technology.

Proof of principle has been provided in a mouse model that homologous gene repair is possible when targeting cells with embryonic properties derived from a somatic cloning procedure; these cells could be clonally selected, expanded and used for rescue of an inborn monogenetic immunodeficiency [1]. However, to translate this revolutionary approach into clinical reality, several limitations remain. These include: the technical difficulties encountered with this technology; the problematic ethical status of human somatic cells that have been reset to an embryonic developmental stage in vitro; issues related to the biological fitness and integrity of such cells [1, 2]; the still low efficiency of homologous gene recombination [1], and finally, the anticipated costs of such procedures.

Therefore, the foreseeable future of corrective gene transfer will still mostly rely on the somewhat suboptimal strategy of somatic gene addition. Using a vector system that mediates stable transgene delivery, recombinant transgene cassettes are introduced into somatic (stem) cells without targeting the mutated allele(s) and without

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Accessible online at:
www.karger.com/aha

Christopher Baum
Department of Hematology and Oncology, Hannover Medical School
Carl-Neuberg-Strasse 1
DE–30625 Hannover (Germany)
Tel. +49 511 532 4523, Fax +49 511 532 4521, E-Mail baum.christopher@mh-hannover.de

further selection of clones with a particular genotype. This reconstitutes a mixed molecular chimerism, in which engineered somatic cells typically vary with respect to the specific transgene insertion site [for review see, 3]. Using appropriate vectors, the vast majority of engineered cells will support sufficient levels of transgene expression. This approach has shown convincing preclinical efficiency in a number of animal models, especially when using well-designed retroviral or lentiviral vectors to introduce transgenes into hematopoietic cells with repopulating capacity. In addition, the field received substantial stimulation by clinical evidence for prolonged correction of two types of severe inherited immunodeficiencies following a retroviral gene-addition strategy [4, 5].

However, some engineered cells may carry unpredictable and potentially dangerous alterations of growth-regulatory genes as a result of random transgene insertion (insertional mutagenesis). Before 2002, such random side effects were anticipated to occur but were not considered as a sufficient single event that could induce a malignant transformation. Just a few weeks after the publication of the encouraging clinical follow-up in the correction of the inherited SCID-X1 immunodeficiency, this long-lasting hope had to be revised. A lymphoproliferative disorder was observed in this paradigmatic clinical trial, and the molecular diagnostics indicated that a well-known oncogene had been activated as a result of random vector insertion [6]. Just a few months earlier, a similar adverse event was observed in a mouse model of genetic cell marking [7]. Technological progress in identifying vector insertion sites of individual cell clones from complex samples was crucial for the rapid recognition of the specific pathogenesis in both cases [8].

These reports induced a worldwide reconsideration of the decision process underlying the preparation and conduction of clinical trials in hematopoietic gene therapy. Some regulatory boards decided to install a moratorium on any clinical trial involving retroviral gene transfer into hematopoietic cells, while others asked for more or less substantial revisions of trial designs and informed consents before continuation [9].

To overcome the current uncertainty in the risk prediction of gene-addition technologies, it needs to be resolved in the near future why the oncogenic risk of retroviral gene addition has not become apparent in more than a decade of clinical trials, which probably involved the manipulation of a huge number of target cells and several hundreds of patients. This may be a consequence of the clinical scenarios and specific transgene vectors involved. As it is likely that the manifestation of severe side effects results from a multifactorial process, it will be of crucial importance to define the significance of individual risk factors. Hence, we have to address the role of vector design, potential side effects of transgene expression (especially when dealing with proliferation-promoting transgenes), as well as the cellular and systemic conditions driving amplification of engineered clones. Therefore, we also need to investigate additional inborn or circumstantial hazards associated with the approaches in which side effects are observed.

Others argue that side effects were observed only recently because most engineered cells introduced in earlier studies were not long-lived enough to establish a proliferating cell clone in vivo. Hence, recent technological improvements in gene transfer protocols would explain why side effects are now observed. Accordingly, reducing the number of repopulating cells exposed to random vector insertion would be one important key to solve the problem. However, this will only provide a solution if safe conditions for engraftment and expansion of a small pool (~ 1,000 to 10,000) of long-lived engineered stem cells are defined.

It appears safe to conclude that the truth lies somewhere in the middle of these considerations. Combined with careful progress and monitoring in clinical gene therapy, ongoing and future research may result in two major advances: a risk classification of technologies and clinical scenarios and, especially for those conditions that remain at increased risk for side effects of gene-addition technology, development of a new hybrid approach that combines random gene addition with targeted clonal selection of somatic stem cells.

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Adoptive Immunotherapy in Chimeras with Donor Lymphocytes

Hans-Jochem Kolb Christoph Schmid Xiao Chen Anja Woiciechowski
Marie Roskrow Martin Weber Wolfgang Guenther Georg Ledderose
Michael Schleuning

Clinical Cooperative Group Hematopoietic Cell Transplantation, Department of Medicine III,
Klinikum University of Munich-Grosshadern, and GSF-National Research Center for Environment and Health,
Munich, Germany

Key Words
Stem cell transplantation · Chimerism · Transplantation tolerance · Adoptive immunotherapy · Donor lymphocyte transfusion

Abstract
Allogeneic stem cell transplantation has a well-defined indication in the treatment of hematological malignancies. The beneficial immune effect of allogeneic marrow transplantation has long been known, but only recently have methods been developed to separate the graft-versus-leukemia (GVL) effect from graft-versus-host disease (GVHD). Animal experiments have shown that lymphocytes from the marrow donor can be transfused without causing severe GVHD if stable chimerism and tolerance is established. First clinical studies have been preformed in patients with recurrent chronic myelogenous leukemia. In these patients complete molecular remissions were induced that persist without further maintenance treatment. These results have been confirmed in larger multicenter studies in Europe and the USA. The best results were obtained in chronic myelogenous leukemia (CML); repeated successes have been reported in relapsing acute myeloid leukemia (AML), myelodysplastic syndromes and multiple myeloma (MMY), and rare responses were reported for acute lymphoid leukemia. Contrary to animal experiments GVHD has been observed in human patients although to a lesser extent than expected in transplants not given immunosuppression. Secondly myelosuppression has been observed in patients treated with relapsing CML. In CML the incidence of GVHD could be reduced by depleting CD8+ T cells from the donor lymphocyte concentrate. Alternatively only small numbers of T lymphocytes can be transfused and in the case of failing responses, the numbers of donor lymphocytes may be increased. Results in recurrent AML have been improved by the use of low-dose cytosine arabinoside, granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor mobilized blood cells as compared to lymphocytes only. In MMY the response rate is higher than in AML, but the remissions are of limited duration in most patients. Several protocols have been designed to include preemptive donor lymphocyte transfusion in patients with a high relapse risk after transplantation. Problems remain to avoid chronic GVHD and to circumvent the immune escape mechanisms of leukemia.

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Prof. Dr. Hans-Jochem Kolb, Hematopoietic Cell Transplantation
José Carreras Transplantation Unit, Klinikum University of Munich-Grosshadern
Marchioninistrasse 15, DE–81377 Munich (Germany)
Tel. +49 89 7095 4240/4241, Fax +49 89 7095 4242
E-Mail Kolb@med3.med.uni-muenchen.de

The success of donor lymphocytes in the treatment of recurrent leukemia has changed the perspectives of hematopoietic stem cell transplantation [1]. The antileukemic effects of myeloablative conditioning have been substituted by adoptive immunotherapy using cells of the marrow donor (fig. 1). Formerly the treatment of leukemia and other neoplastic diseases of the hematopoietic system focused on the maximal tolerated dose of radiation and chemotherapy to destroy the leukemia as much as possible and to rescue the patient from hematopoietic failure by transplanting bone marrow. Today the limitations and risks of high-dose chemotherapy and radiotherapy are well known and the conditioning treatment is designed to allow the establishment of chimerism and the development of transplantation tolerance as a platform for immunotherapy. The conditioning does not need to be myeloablative. There is a therapeutic dilemma in bone marrow transplantation for malignant diseases. Acute graft-versus-host disease (GVHD) and its sequelae are the major complications of allogeneic stem cell transplantation. The most effective method to prevent GVHD is the depletion of T lymphocytes from the transplant [2, 3]. However, the depletion of T cells from the graft ablated most of the antileukemic effect of allogeneic transplantation [4]. Adding back small amounts of T cells to the depleted graft was not successful in reducing the risk of relapse without inducing GVHD. However, transfusion of donor lymphocytes into stable canine chimeras did not produce GVHD [5]. Therefore in the 1980s we studied donor lymphocyte transfusions (DLTs) in canine chimeras with the aim of influencing chimerism and transfer immunity from the donor to the host [6].

The animal experiments encouraged us to use donor lymphocytes for the treatment of relapse of chronic myelogenous leukemia (CML) in 3 patients [7]. The results were confirmed by several single centers [8–14], and the spectrum of graft-versus-leukemia (GVL) activity was assessed in multicenter analyses [15, 16]. Native donor lymphocytes and sensitized T cells, T-cell lines and clones were successful in the treatment of viral infections [17–19]. Similar strategies have been explored using minor histocompatibility antigens with restricted tissue expression in the treatment of leukemia [20–22]. Here we review the current status of adoptive immunotherapy with donor cells and we try to give a perspective to the future of immunotherapy.

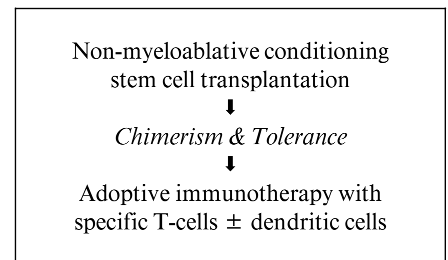

Fig. 1. Strategy of adoptive immunotherapy in chimeras. Adoptive immunotherapy with T cells with or without dendritic cells allows non-myeloablative conditioning. As soon as chimerism is stable and immunosuppression can be discontinued, a state of immunological tolerance is established. At this time donor cells can be transfused without severe GVHD.

Principles Derived from Animal Studies

There have been many studies on murine leukemia which differs in several aspects from human leukemia. Bacteria-free mice with spontaneous AKR leukemia have been treated with some success using marrow and low-dose lymphocytes from major histocompatibility complex (MHC) mismatched donors [23].

The concept that leukemia expresses minor histocompatibility antigens on the hematopoietic cells of the host was the starting point for the production of mixed DLA-identical chimeras in dogs. Conversion of mixed chimerism into complete chimerism served as a model for a GVL reaction. Mixed chimeras were produced by transplantation of low numbers of marrow cells depleted of T cells by the treatment with absorbed antithymocyte globulin (ATG) to prevent GVHD. These animals were stable mixed chimeras. Transfusion of donor lymphocytes on days 1 and 2, or days 21 and 22 after marrow transplantation induced fatal GVHD. However, transfusion on days 61 and 62 did not produce GVHD and the animals survived. These animals were mixed lymphoid and myeloid chimeras prior to transfusion and they became complete chimeras thereafter [6]. The donors were immunized against tetanus toxoid and the recipients developed antibody titers after DLT that persisted for more than 3 years after booster injections. Transfused and nontransfused animals were immunized against diphtheria toxoid as a new antigen. Transfused dogs developed significantly higher antibody titers than nontransfused dogs.

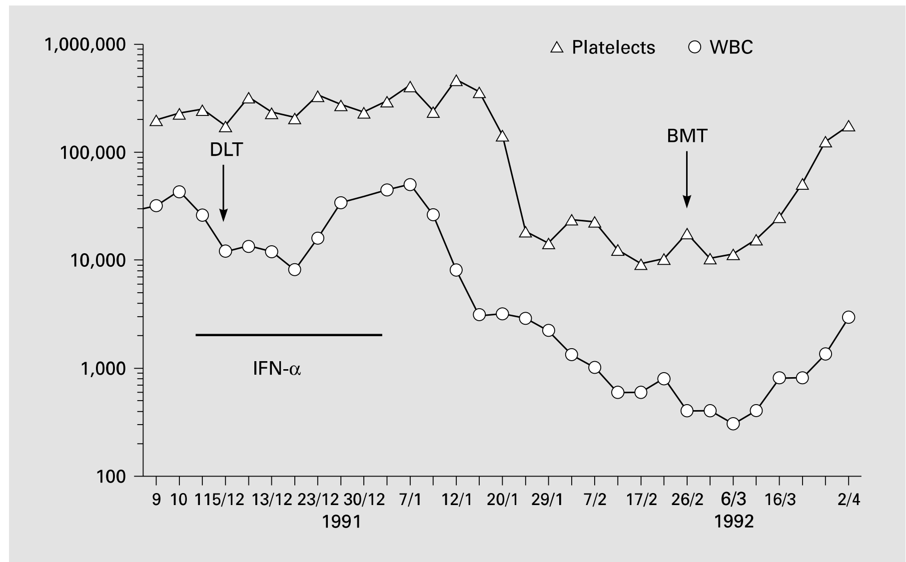

Fig. 2. The course of a 39-year-old patient suffering from myelosuppression following DLT. After DLT WBC increased and the patient developed fever until the blood counts dropped and the patient became severely pancytopenic. Transfusion of marrow from his donor without further conditioning resulted in complete restoration of hematopoiesis and complete chimerism. A molecular remission was found after marrow transfusion that persists until presence.

In mice a delay of 3 weeks for the transfusion of donor lymphocytes was enough to prevent GVHD [24].

The importance of the delay of DLT is obvious, but the cause of GVHD is not clear. One possibility is the 'cytokine storm' set free by the conditioning treatment with radiation and chemotherapy [25] that may have settled after 3 weeks and 2 months, respectively. Another possibility is the establishment of peripheral tolerance maintained by donor T cells in collaboration with host dendritic cells. The latter mechanism is supported by the finding that depletion of donor lymphocytes in the chimera prior to DLT predisposes the recipient to vigorous GVHD (Menzel H, 1996, unpublished) [26]. In man the necessary delay is not known. It may vary with age and previous chemo- and radiotherapy.

Results of DLTs in CML

Three patients with recurrent CML after allogeneic marrow transplantation were treated with DLT in 1988 and 1989 [27], they are still in hematologic and molecular remission of CML. Acute GVHD developed in 2 patients requiring immunosuppressive treatment, and chronic GVHD in 1. Immunosuppressive treatment could be discontinued in both. Severe myelosuppression was observed in a 4th patient treated 1991. Pancytopenia occurred 2 months after DLT and it did not respond to treatment with granulocyte colony-stimulating factor, but to the transfusion of donor marrow (fig. 2).

The analysis of the results of centers of the European Cooperative Group of Blood and Marrow Transplantation (EBMT) showed best results in cytogenetic and hematologic relapses of CML, intermediate results in transformed phase CML, acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS), and poor results in acute lymphoblastic leukemia (ALL) [15]. Single patients with chronic myeloproliferative diseases as polycythemia vera and myeloid fibrosis [28] also responded to DLT. Both the absence of chimerism [29] and the presence of GVHD at the time of DLT were adverse factors for a response. In CML the GVL effect correlated with the severity of GVHD, but responses were also seen in patients without GVHD. However GVL was limited to patients with an allogeneic donor, it failed in patients with a monozygotic twin donor. The time until molecular remission was between 4 and 6 months after a single transfusion in most patients; in some patients molecular remissions were reached after more than a year (fig. 3). Antigen presentation could be improved by treatment with cytokines. In particular the combination of interferon-α (IFN-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) improved the expression of class-I and II human lymphocyte antigens (HLAs), CD40 and CD80 [30]. Preliminary results confirm the beneficial effect of GM-CSF and IFN-α in patients with recurrent CML refractory to donor lymphocytes.

Complications of the treatment were GVHD and myelosuppression. Myelosuppression was more frequent in hematological relapse than in cytogenetic relapse. The

use of mobilized blood cells containing stem cells instead of lymphocytes did not prevent myelosuppression [31]. Prevention of GVHD could be achieved by two methods without ablating the GVL effect: depletion of CD8+ T cells from the transfusion [32, 33], and using escalating doses of DLT [34] starting at 2×10^6 lymphocytes/kg. The escalating dose schedule has significantly lowered the risk of GVHD [35]. Patients should be surveyed by regular quantitative reverse transcriptase polymerase chain reaction for bcr/abl, and in case of persisting or recurrent positivity the proposed schedule is started at a dose of 2×10^6 lymphocytes/kg from unrelated donors and 1×10^7 lymphocytes/kg from an HLA-identical sibling donor. Doses are escalated if there is no GVHD within 30 days or no response within 60 days.

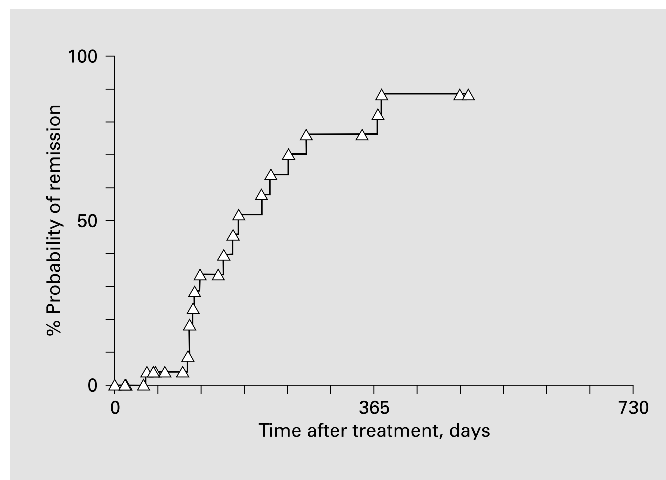

Fig. 3. Time to molecular remission after DLT. Molecular remissions were achieved after 4 months, the median time was 6 months and late remissions occurred after more than 1 year. Data are from the Transplant Center Munich Grosshadern, evaluated in March 2001.

Results of DLTs in AML and MDS

The EBMT results indicated inferior responses in patients with recurrent AML after DLT. In patients without chemotherapy-induced remission the response rate was 25% with very few patients surviving more than 4 years. In a second analysis of 120 patients with AML and MDS reported to the EBMT, complete remissions could be induced in 45 (41.6%) of 108 evaluable patients including patients treated with chemotherapy and DLT [36]. The median duration of remission was 304 days, in 18 patients remissions lasted more than a year, and in single patients more than 5 years. Overall survival was greater in responding patients. Three risk factors could be identified as being associated with a poor response to DLT: a short remission after allogeneic transplantation of less than the median of 194 days ($p = 0.02$); withholding chemotherapy prior to DLT ($p = 0.001$), and the absence of acute GVHD of grade II or higher after DLT ($p < 0.0001$). Of patients without GVHD only 18% responded as compared to 66% of patients with GVHD > grade I. Patients with late relapse after transplantation responded more frequently (48%) than those with early relapse (28%). After DLT the complete remission rate was independent of gender and age of the patient and donor, their relationship, the number of cells transfused and whether or not T-cell depletion was used for prophylaxis of GVHD after transplantation. Survival of patients without complete remission was poor, but once a complete remission was achieved survival was not different whether or not the remission was induced by chemotherapy. This finding supports the hypothesis that DLT maintains the remission and favors the use of chemotherapy for remission induction.

Only limited data were available on the FAB subtype and cytogenetic analyses in these patients. With these limitations neither the FAB subtype nor the karyotype influenced the response.

Poor antigen presentation and the rapid progression of the disease were considered as the major obstacles for adoptive immunotherapy in recurrent AML. Improvement of antigen presentation and production of cytotoxic T cells against autologous blasts was studied in vitro (fig. 4). The combination of GM-CSF, IL-4, TNF-α and FLT3-L was particularly effective in inducing dendritic cells from AML blasts [37]. The culture was effective in 77% of patients and included patients with unfavorable karyotypes. Specific cytotoxic T cells against autologous blasts could be produced in more than 60% of these patients.

In a recent study we have used low-dose cytosine arabinoside as mild chemotherapy for halting progression of the disease and GM-CSF for improving antigen presentation. Mobilized blood (MDBC) was transfused as a preparation of stem cell-enriched donor lymphocytes and GM-CSF was applied for 14–28 days after transfusion. This way antigen presentation was optimized by induction of dendritic cells from AML blasts and substitution of dendritic cells derived from CD34+ cells of the graft. The response rate was improved from 25 to 67% and the actuarial probability of survival is 25% at 4 years [38]. In

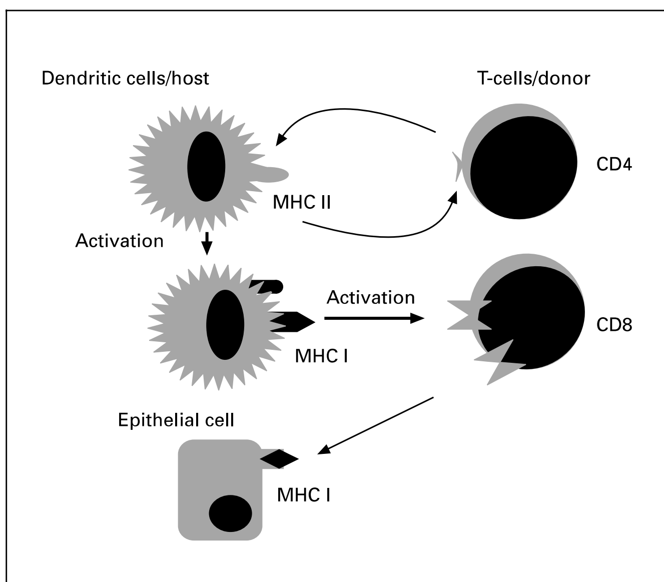

Fig. 4. Pathophysiology of GVHD. There is mutual activation of donor CD4+ T cells and host dendritic cells which may be accelerated by pre-activation of dendritic cells by radiation, chemotherapy, endotoxin from intestinal flora, viral infections and by pre-immunization on the side of the T cells against minor histocompatibility antigens, viral antigens and others. Activated dendritic cells activate CD8+ T cells and present HLA class-I-restricted peptides to CD8 T cells which become activated and react against normal cells of the host.

some responding patients the treatment was repeated after 4–6 months, and the patients have remained in remission.

In patients with progressive disease after MDBC, low-dose cytosine arabinoside and the transfusion of donor T cells has been used with success, but GVHD was mostly severe.

GVHD and extramedullary relapses remain therapeutic problems. In most patients with extramedullary relapse and some patients with systemic relapse, low-dose cytosine arabinoside is not effective in halting disease progression. In these more intensive chemotherapy including anthracyclins is necessary; solitary infiltrates may be radiated prior to transfusion of donor cells [39]. Following more intensive chemotherapy severe GVHD may develop after transfusion of mobilized blood cells and treatment with GM-CSF. In these cases GM-CSF has to be stopped and immunosuppressive treatment with steroids, cyclosporin A and azathioprine or others has to be started. GM-CSF should also be discontinued if blasts are mobilized from the marrow into the blood. Unfortunately leukemia may recur during immunosuppressive treatment and few therapeutic options remain.

Results of DLTs in Myeloma

The best responses next to CML were seen in recurrent multiple myeloma [40–42]. The most sensitive marker for response is the monoclonal paraprotein, next are infiltrates of plasma cells in the marrow and the disappearance of lumps, and the least sensitive are osteolytic lesions. The time to response may be 4–6 months or longer, and unlike in CML hematological remissions are less likely to be complete [43]. In most instances remissions are not as durable as in CML, but durable partial remissions have been observed in single patients (Kolb, unpublished). GVHD is observed in most responding patients, it may even recur after chemotherapy for myeloma [44]. Prevention of GVHD by depletion of CD8+ T cells from DLT was used with some success [33], repeated low doses of unseparated DLT were also effective [45].

Preemptive treatment with DLT may improve the outcome in combination with T-cell depletion, but the optimal strategy has not been found [46]. Immunization of a donor against the idiotype of the myeloma and the transfer of a cellular proliferative response has been reported [47], but the reactive donor T cells were found in the patient with persisting paraprotein.

Results of DLTs in Other Diseases

The response of recurrent ALL to DLT was poor in most cases [5, 48], but there are exceptions with long-lasting remissions [49, 50]. The first case of Slavin et al. [13] was a child with ALL who received donor cells 4 weeks after transplantation for residual leukemia. Remissions have been described in patients with Hodgkin's disease, non-Hodgkin's lymphoma [51] and chronic lymphocytic leukemia, but the overall response is controversial.

Neoplastic diseases other than hematological have been treated with allogeneic transplantation and DLT with some success. Metastatic renal cell cancer has shown sustained responses [52], some response was also observed in breast [53] and ovarian cancer (Kolb unpublished).

Non-malignant diseases have benefited from DLT in cases of poor graft function after non-myeloablative conditioning [54]. Allogeneic transplantation and DLT have been advocated for the treatment of autoimmune diseases

[55], since patients with autoimmune disease who had been transplanted for leukemia were cured of the autoimmune disease in most instances. However, chronic GVHD may complicate allogeneic stem cell transplantation after non-myeloablative conditioning with symptoms similar to those of autoimmune diseases.

DLTs have been used for the treatment of viral infections after transplantation, in particular Epstein-Barr virus-induced lymphoproliferative disease [18]. In these cases minute numbers of T cells were sufficient and the reactions were associated with an acute inflammatory response. Pre-immunized T-cell lines were better tolerated and effective [19]. Adoptive immunotherapy of viral infections has shown promising results which may lead to better understanding of the GVL response.

Mechanism of the GVL (Tumor) Effect

The absence of a measurable GVL effect in patients with syngeneic twin donors indicates the importance of an alloimmune response [15]. Minor histocompatibility antigens are expressed on leukemia cells and can serve as targets for a GVL effect without GVHD, if their expression is restricted to hematopoietic tissue [22, 56]. HA-1 and HA-2 are such peptides with restricted expression to the hematopoietic system, but Y-associated minor histocompatibility antigens are also candidates [57, 58]. Tissue-restricted expression of minor histocompatibility antigens may be operationally limited to the hematopoietic system by presentation of class-II HLAs [59].

The effector cells of the GVL reaction are not well defined. CD4+ T cells might be candidates, since CD8+ T cells could be depleted without losing the GVL effect [32]. However CD4+ T cells can recruit CD8+ T cells and other cells in vivo [60].

There is good evidence that ex vivo T cells immunized against minor histocompatibility antigens effectively lyse leukemia cells in vitro [21, 61] and in immunodeficient mice in vivo [62]. However, ex vivo immunized T cells have not yet been used widely in human patients. The most convincing example of leukemia treatment with immunized cells is the trial of Falkenburg et al. [63] who selected cytotoxic T cells on the basis of their reactivity to CML cells and infused them repeatedly into a patient with accelerated phase CML. Slavin et al. [64] immunized donor cells with cell lysates of the parents and found a complete response in a patient with accelerated phase CML who had not responded to DLT.

Antigens other than minor histocompatibility antigens that may be candidates for a GVL effect comprise fusion peptides, peptides from proteins encoded by mutated genes and proteins of overexpressed genes. Products of disease-specific rearranged genes as BCR/ABL in CML, PML/RARα in AML FAB M3 and AML1/ETO and others contain highly specific fusion peptides. Proteins of genes with point mutations as in RAS genes and peptides of overexpressed normal genes such as p53 and proteinase 3 [65] have been studied. The antibody idiotype of a lymphoma and myeloma could also be seen as an overexpressed normal protein that marks the tumor. T-cell reactivity has been described for all of these, but only BCR/ABL-specific T cells recognize malignant cells in patients, and T cells with that specificity are occasionally found in patients [66]. To date none of these antigens has been used successfully for adoptive immunotherapy in chimeric patients [67].

Our hypothesis that because myeloid leukemia produces dendritic cells of leukemia origin it responds better to DLT than lymphoid leukemia has been supported by the finding that dendritic cells in CML carry the bcr/abl translocation [30, 68, 69], and AML cells differentiated the karyotypic marker to dendritic cells [37, 70] in fluorescent in situ hybridization (FISH).

The production of dendritic cells from leukemia precursors has been studied in vitro because most cases of AML, as lymphomas and lymphoblastic leukemia, do not express co-stimulatory molecules such as CD80 and CD86. Untreated CML is poorly stimulatory in mixed lymphocyte reactions and fails to induce cytotoxic T cells in many instances [71]. Culture of AML blasts in the presence of GM-CSF, IL-4 with or without TNF-α and FLT3-L induces the expression of co-stimulatory molecules [37] (fig. 5). Similarly culture of CML cells in the presence of IFN-α and GM-CSF stimulates expression of co-stimulatory molecules and the generation of cytotoxic T lymphocytes [72]. The combination of GM-CSF and IFN-α has already been used successfully in patients with relapse of CML not responding to DLT alone and DLT plus IFN-α (unpublished). Other cytokines such as IL-2 have been introduced on the basis of animal experiments [73]. IL-2 may support the T-cell reactivity after immunization. In myeloma and lymphoid neoplasms stimulation of donor T cells is poor and new ways to improve specific T-cell stimulation are being explored. One possibility is the transfer of genes coding for immunostimulatory molecules and proinflammatory cytokines [74]. Other ways are the transfusion of dendritic cells with the DLT or the use of MDBC together with the treatment with GM-CSF.

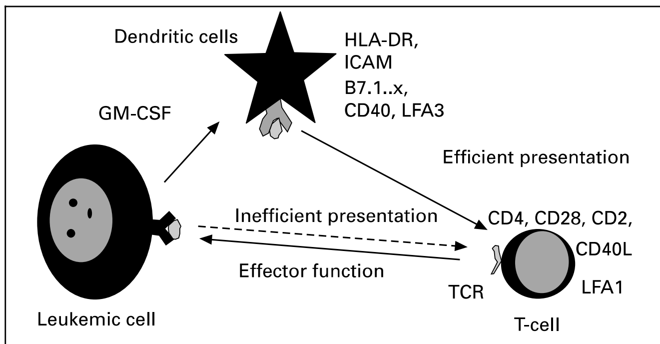

Fig. 5. AML cells may develop to antigen-presenting cells. AML blasts are deficient of co-stimulatory molecules and are thus inefficient in the presentation of antigen to donor T cells. Treatment with GM-CSF drives blasts to express the stimulatory molecules necessary for efficient antigen presentation. These T cells then react against residual blasts.

Table 1. Possible mechanisms of immune escape

Possible mechanisms of immune escape
Mechanisms on antigen presentation
Low expression of co-stimulatory molecules: B7.1, B7.2, CD40, ICAM
Downregulation of HLA class I, class II antigens or relevant alleles
Inefficient peptide production by proteasome/TAP mechanism
Secretion of inhibitory cytokines as IL-10, TGF-β
Low secretion of proinflammatory cytokines TNF-α and IFN-γ
Expression of FAS-L on tumor/leukemia cells inducing apoptosis of T cells
Expression of nonfunctional FAS on leukemia blasts
Mechanisms on T cells
Downregulation of ζ-chain (lymphoma and CML) and ε-chain (CML) of the T cell receptor
Downregulation of CD28 in AML
Others

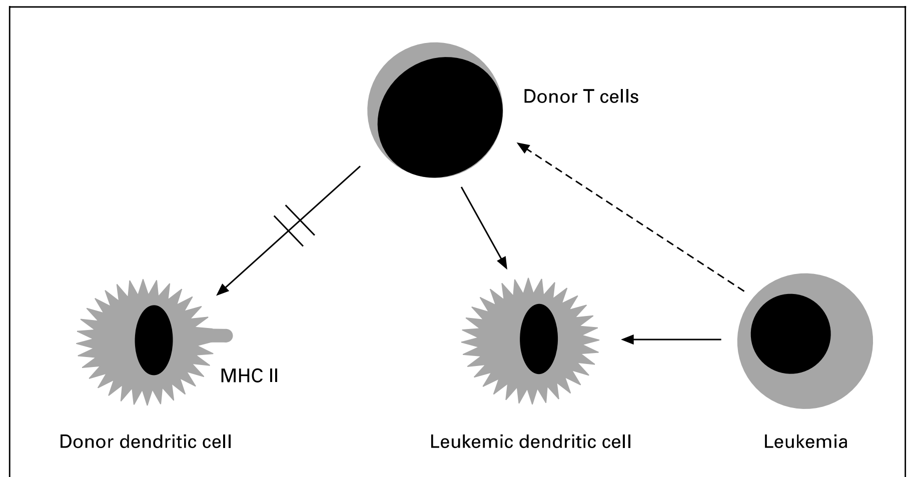

Fig. 6. Mechanism of the graft-versus-leukemia reaction. After establishment of chimerism dendritic cells of the host are replaced by dendritic cells of the donor. Leukemic blasts are potentially the remaining hematopoietic cells of the host which may become stimulatory and a target for donor T cells, if they are driven towards dendritic cells. The reaction is specific because minor histocompatibility antigens with restricted expression are involved and donor dendritic cells maintain tolerance in other organs.

Immune reconstitution after DLT has been studied with typing for T-cell receptor V-β families [75] and T-cell receptor excision circles [76]. DLT enhanced immune recovery and converted to full donor chimerism. In single patients with GVL reactions clonal T-cell restitution has been observed [75]. T cells of the donor produced similar clones when exposed to the leukemia in vivo as in vitro, but genetic analysis showed that they were different [77].

Intercurrent infections with viruses or other intracellular microorganisms may jeopardize the result of DLT. Most patients experiencing viral infections in the first weeks after DLT develop GVHD. A similar observation has been reported in patients given T-cell-depleted transplants and DLT. CMV seropositivity of the patient was the most significant risk factor for survival [78]. It is being discussed whether anti-infectious prophylaxes should be given to patients after DLT.

There are numerous mechanisms how neoplasms and leukemia may escape an immune reaction against themselves (table 1) [79]. Most importantly the expression of antigens on the cell surface may change. Even the loss of the Philadelphia chromosome has been reported [80]. In this context the use of minor histocompatibility antigens is most promising, because in the chimera most hematopoietic cells are substituted by the donor and only leukemia cells are still of host type (fig. 6).

The role of natural killer (NK) cells has always been debated in allogeneic stem cell transplantation. During

post-transplant recovery NK cells are early and trials of substitution early after transplantation have not been successful. In murine models allogeneic NK cells were effective [81], but depletion of CD3+ T cells abrogated the effect [82]. In HLA-mismatched transplantation complete T-cell chimerism is not required for GVL to occur, mixed chimeras can be cured of lymphoma [83]. In mixed chimeras DLT exert a strong effect against lymphoma without causing GVHD [84]. The role of NK cells has recently been investigated in HLA-mismatched transplantation and excellent results were reported in AML, if donor and patient differed in the killer inhibitor receptors [85]. NK cells are inhibited from killing if the target cells share certain HLA antigens. In HLA-mismatched transplantation NK cells of the donor can kill leukemia cells and at the same time suppress rejection of the transplant. In the case of the donor and patient belonging to a different alloreactive group, relapses of AML did not occur.

Outlook for Adoptive Immunotherapy

Hematopoietic cell transplantation has come a long way from bone marrow transplantation to adoptive immunotherapy in chimeras. However, the mechanisms of adoptive immunotherapy in chimeras are still far from being understood. The immunobiology of leukemia, other neoplasia and viral infections has to be studied further in human patients. The mechanism of immune tolerance, immune reactivity against normal cells, and transfer of immunity can be studied in animal experiments.

Immunization of donor T cells against minor histocompatibility antigens of the recipient is currently studied in the dog. Sensitized cells convert mixed to complete chimerism much faster than naive T cells. Tests have been developed to demonstrate cellular immunity to hematopoietic progenitor cells in vitro allowing the definition of minor antigens in the dog [86]. The incidence of severe GVHD after transfusion of sensitized donor lymphocytes into stable chimeras may be 30–50% [5]. The percentage is expected to be higher in humans since patients and their donors are exposed to a multiplicity of histocompatibility and viral antigens during their life. Preventive measures against severe GVHD are necessary. Modification of donor lymphocytes with a suicide gene is the most promising way of prevention. T cells of the donor are infected with a replication-deficient retrovirus carrying the herpes simplex thymidine kinase gene (HSV-Tk) which can phosphorylate ganciclovir and the resulting nucleotide leads to the stop of DNA polymerization during cell division [87]. Current problems of the method are altered immune reactivity of transduced T cells, immune reaction against the viral protein and rejection of the transduced cells and altered sensitivity of transduced cells to ganciclovir due to splice variants of the gene. Recently the development of leukemia has been reported in a mouse treated with cells carrying the marker gene (a truncated nerve growth factor receptor) without the suicide gene. We have studied the method in the dog and found a good immune reactivity of transduced canine T cells in vitro. Transfusion of transduced T cells into a canine chimera resulted in a complete chimerism and transfer of immunity to tetanus toxoid [88].

Adoptive immunotherapy in chimeras is a promising way to treat leukemia and possibly solid neoplasia. In particular the immune reactivity against leukemias and neoplasia otherwise refractory to chemotherapy gives new perspectives in hematology and oncology. Several leukemia study groups have included preemptive DLT as prophylaxis in high-risk leukemia according to the schema shown in figure 1. The results are pending.

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Cellular Engineering of HSV-tk Transduced, Expanded T Lymphocytes for Graft-versus-Host Disease Management

Scott R. Burger[a] Diane M. Kadidlo[b] Lisa Basso[c,d] Nancy Bostrom[b]
Paul J. Orchard[c,d,e]

[a]Advanced Cell and Gene Therapy, Chapel Hill, N.C. and Department of Laboratory Medicine and Pathology, University of Minnesota Medical School, [b]Cell Therapy Clinical Laboratory, Fairview University Medical Center, [c]Program in Blood and Marrow Transplantation, [d]Department of Pediatrics, [e]Institute of Human Genetics, University of Minnesota Medical School, Minneapolis, Minn., USA

Key Words
HSV-tk · Suicide genes · Allogeneic T lymphocytes · Graft-versus-host disease · Cell therapy

Abstract
Engineering donor T lymphocytes with inducible 'suicide genes', such as herpes simplex virus thymidine kinase, has potential to improve safety and efficacy in allogeneic transplantation by facilitating management of graft-versus-host disease. Elective administration of a relatively nontoxic pro-drug would induce in vivo negative selection of engineered lymphocytes specifically, sparing other donor hematopoietic cells. The engineered cells must retain immunologic function, and undergo negative selection in response to clinically attainable plasma concentrations of pro-drug. The cell engineering process itself, typically involving activation, transduction, ex vivo expansion, and selection, must produce clinically useful numbers of genetically modified cells at high purity. We discuss development of a cellular engineering manufacturing process that yields transduced, expanded T lymphocytes meeting these requirements.

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Introduction

Morbidity and mortality associated with graft-versus-host disease (GVHD) remain a major limitation of allogeneic hematopoietic cell transplantation [1, 2]. While the incidence and severity of GVHD can be decreased by depletion of T lymphocytes from the allogeneic graft, this process has been associated with increased risk of graft failure, and may compromise both immune reconstitution [3–5] and the beneficial graft-versus-leukemia (GVL) effect [6–8], with corresponding increased risk of relapse.

An alternative approach to T-lymphocyte depletion for GVHD prevention is to provide allogeneic donor T lymphocytes with the graft to facilitate engraftment and GVL, while managing GVHD by other means. The molecular engineering of cells with 'suicide genes' has the potential to inhibit the proliferation of or eradication of cells expressing the gene in the presence of an otherwise relatively nontoxic pro-drug. The expression of these genes in donor T cells has promise in GVHD management, as they may be specifically eliminated in vivo by administration of the appropriate pro-drug, while sparing other hematopoietic cells within the graft.

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Fax + 41 61 306 12 34
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Scott R. Burger, MD
Advanced Cell & Gene Therapy
105 Highgrove Drive
Chapel Hill, NC 27516 (USA)
Tel./Fax +1 919 969 1103, E-Mail sburger@ac-gt.com

Several suicide gene/pro-drug combinations have been studied, including cytosine deaminase/5-fluorocytosine [9], P450–2B1/cyclophosphamide [10], bacterial nitroreductase/CB1954 [11], and more unusually, a Fas-FKBP chimera which induces apoptosis in response to bivalent FKBP ligand [12]. The herpes simplex virus thymidine kinase suicide gene (HSV-tk) is, however, undoubtedly the most extensively studied for use in cellular therapy. Cells transduced with the HSV-tk gene phosphorylate the pro-drug ganciclovir (GCV) to the monophosphate form, which is subsequently converted by endogenous kinase activity to GCV-triphosphate, which inhibits DNA synthesis.

Using this strategy, the administration of GCV therefore has the potential to selectively eliminate HSV-tk expressing cells in vivo, and therefore to control undesirable T-lymphocyte activity, specifically GVHD mediated by transduced T lymphocytes. Administration of allogeneic donor T lymphocytes transduced with such a suicide gene, administered in the setting of a T-cell-depleted hematopoietic cell transplant, would take advantage of the positive effects of donor T lymphocytes on engraftment, GVL and immune function, while providing an increased measure of safety due to the potential to eliminate T lymphocytes in vivo if severe GVHD is observed [13–16].

Cell Engineering Requirements for T-Lymphocyte Suicide Gene Therapy

For this T-lymphocyte suicide gene strategy to be practical clinically and provide likelihood of success, the cell engineering process and the lymphocyte product must meet certain basic requirements (table 1). The engineered donor lymphocytes must retain immunologic function as close as possible to unmanipulated cells to provide immune reconstitution, facilitation of engraftment, and GVL, despite undergoing the extensive cell processing and gene transfer necessary for their production, as this therapeutic strategy is based on exploiting the beneficial functions of allogeneic lymphocytes after transplantation. Second, the engineered lymphocytes must be sensitive to the pro-drug at plasma concentrations that are attainable in transplant patients, with appropriately complete and definitive negative selection. This, too, is fundamental to the strategy of suicide gene therapy, as the ability to perform in vivo negative selection of the engineered lymphocytes by administering the pro-drug is to provide the principal protection against the morbidity and mortality of GVHD. Third, if the suicide gene is to be transferred and expressed by means of a retroviral vector, then the target lymphocytes must be actively proliferating [17]. While alternative means of gene transfer exist, including nonviral strategies, the most common method of clinical gene transfer in hematopoietic cells remains retroviral-mediated gene transfer, which requires activation prior to transduction to achieve efficient transduction. Fourth, the donor T lymphocytes administered must not contain significant numbers of nontransduced lymphocytes, which would not be controllable by pro-drug-induced negative selection. As gene transfer remains an inefficient process, a post-transduction positive selection step is likely to be required in which transduced cells are selected to high purity. Because the suicide gene itself does not provide a basis for positive selection, a second gene providing expression of a selectable marker is typically provided on the vector in addition to the suicide gene.

Table 1. Process requirements for T-lymphocyte suicide gene therapy

Engineered lymphocytes retain sufficient immunologic function to provide enhanced immunologic reconstitution, facilitation of engraftment, and GVL in comparison to a T-cell-depleted graft
Engineered lymphocytes demonstrably sensitive to pro-drug at levels achievable in vivo
Gene transfer frequency sufficient to provide adequate numbers of engineered cells. If retroviral-mediated gene transfer is used, an activation step is required to initiate proliferation prior to transduction
Process yields engineered lymphocytes at high purity and in sufficient numbers for clinical application

These four requirements define much of the cell engineering process for generation of suicide gene-transduced lymphocytes (table 2). For protocols using retroviral-mediated gene transfer, lymphocytes must be enriched or isolated from allogeneic donor blood, activated to induce proliferation and transduced with a vector providing the capacity for both positive and negative selection [18]. Following transduction, the engineered cells must undergo enrichment based on expression of the positive selectable marker. A period of cell expansion in the process assists in attaining a sufficient dose of engineered lymphocytes. By performing cell expansion after transduction and prior to selection, one may minimize the quantity of vector required as well as expand the transduced population, thereby increasing the efficiency and recovery of the cell selection step [18]. Additional cell expansion after selec-

Table 2. Cell engineering process outline for lymphocyte transduction with suicide gene and selectable marker, expansion and selection of engineered lymphocytes

Process step	Process elements	Questions
Day 0 Cell proliferation	Anti-CD3/CD28 beads IL-2: 1,000 U/ml	Optimal bead:target ratio Duration of incubation with CD3/CD28 beads Optimal IL-2 concentration Duration of culture in IL-2
Day 2 Transduction	MFG-TKiNG vector Protamine Centrifugation	Centrifugal force Timing after activation Transduction duration Recovery after transduction
Days 3–14 Cell expansion	Culture × 11 days X-VIVO-15 culture medium IL-2: 1,000 U/ml CD3/CD28 beads from day 1 activation step	Protein source (FFP, FBS, HSA) Medium changes Culture duration Optimal IL-2 concentration, if any Duration of exposure to CD3/CD28 beads
Day 14 Anti-CD3/CD28 bead removal Transduced cell selection	NGFR+ cell selection Anti-NGFR biotinylated antibody Anti-biotin magnetic particles	Optimal selection device: Miltenyi CliniMACS or Nexell Isolex 300i

Donor lymphocytes were transduced with a thymidine kinase suicide gene and a gene encoding a truncated nerve growth factor receptor (NGFR), then expanded and selected for NGFR+ cells.

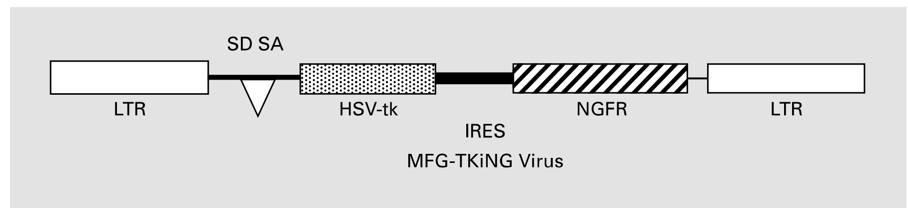

Fig. 1. MFG-TKiNG retrovirus. The herpes simplex virus thymidine kinase gene (HSV-tk) and the truncated nerve growth factor receptor (NGFR) are co-expressed using the internal ribosome entry site (IRES) from the encephalomyocarditis virus. Splice donor (SD) and splice acceptor (SA) sites are designated, as well as the retroviral long terminal repeats (LTR).

tion also could be considered, depending on the purity and yield of the engineered lymphocytes following selection.

Several means exist for providing positive selection of a heterogeneous population of transduced and untransduced cells. Antibiotic genes providing resistance to neomycin and hygromycin have been used, but require an extended culture period. In addition, there is evidence that the neomycin phosphotransferase gene is immunogenic [19]. An alternative approach for positive selection is based on expression of a cell surface molecule that may be bound by specific antibodies. An example is the human nerve growth factor receptor (NGFR) gene, which is expressed on NGFR-transduced human T cells [20]. This strategy permits both enrichment of transduced cells to high purity using immunomagnetic bead technology, as well as monitoring of the persistence of transduced cells in vivo by flow cytometry [13]. For these reasons, the truncated NGFR gene was incorporated into the construct design for the HSV-tk gene vector (fig. 1).

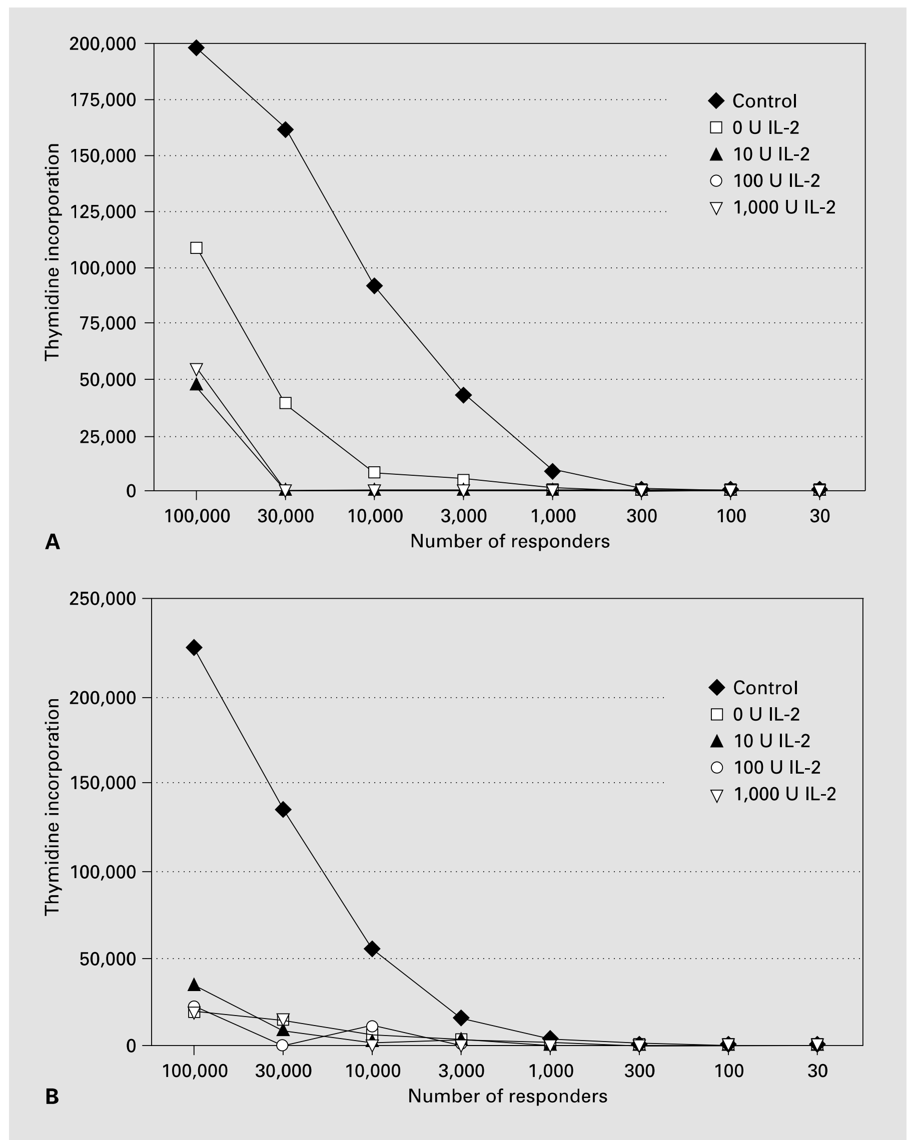

Fig. 2. Effect of IL-2 on PHA-induced proliferation of anti-CD3/CD28-activated cells following a secondary PHA stimulus. Donor-derived MNC were obtained, and a sample frozen prior to activation (control). Additional cells were activated with anti-CD3/CD28 beads in X-VIVO-15 medium in concentrations of IL-2 from 0 to 1,000 U/ml and expanded for 14 days. A sample of cells were removed after 7 days in culture (**A**) or after 14 days in culture (**B**) and tested to determine their response to a secondary stimulus of PHA (10 μg/ml). In proliferation assays semi-log dilutions of cells from 10^5 to 30 cells/well were placed in a total of 200 μl of medium in 96-well plates, and were pulsed on day 6 for 16 h with 1 μCu of [^{3}H]-thymidine (Perkin Elmer Life Sciences Inc., Boston, Mass., USA) prior to cell harvesting and determinations of thymidine incorporation (cell harvester, matrix 9600 reader; Packard Instruments, Downer's Grove, Ill., USA).

An effective process for generation of a pure population of transduced lymphocytes requires process evaluation and testing to establish optimal conditions and specifications. Table 2 includes an outline of the initial process used to generate HSV-tk- and NGFR-transduced, expanded lymphocytes in our cell-engineering laboratory, as well as some of the process development questions encountered.

Lymphocyte Activation, Proliferation

To achieve high efficiency retroviral-mediated gene transfer it is critical to induce the target cell population to undergo mitosis to facilitate integration [17, 21]. Use of anti-CD3 antibodies and IL-2 has been shown to generate large numbers of T cells [22, 23], and has been used to provide transduced T cells for clinical use [15]. There is, however, reason for concern that use of antibodies capable of cross-linking the T-cell receptor alone may induce eventual apoptosis of the activated cells.

We performed lymphocyte activation using anti-CD3/CD28 antibodies bound to magnetic beads designed to

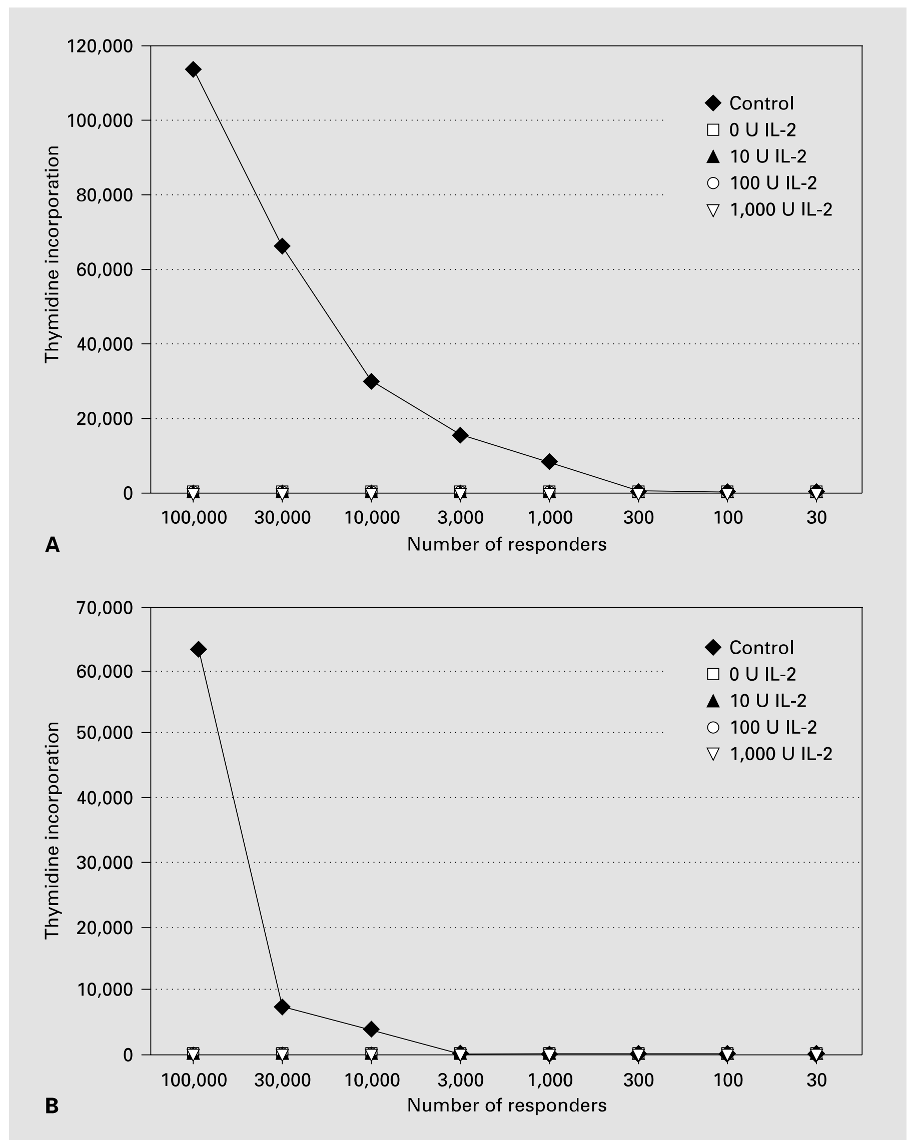

Fig. 3. Effect of IL-2 on allogeneic responses of anti-CD3/CD28 activated cells. Donor-derived MNC were activated with anti-CD3/CD28 beads and cultured in IL-2 from 0 to 1,000 U/ml as in figure 2. On day 7 in culture a sample of cells were tested in a mixed lymphocyte reaction (MLR) assay to determine their ability to respond to allogeneic stimulation. The assay was also performed on cells maintained in identical culture conditions until day 14 after activation. Dilutions of responder cells (10^5 to 30 cells/well) from day 7 (**A**) or 14 (**B**) were placed in culture with 50,000 irradiated stimulator cells, and pulsed on day 6 for 16 h with 1 μCi of [^{3}H]-thymidine prior to cell harvesting and determinations of thymidine incorporation.

cross-link the T-cell receptor and co-stimulatory pathways simultaneously [24, 25]. This approach would be expected to initiate proliferation of T cells [26–28], with potential to decrease development of anergy or apoptosis associated with stimulation through the T-cell receptor alone [29–32].

The initial procedure specified performing lymphocyte activation for 48 h in the presence of IL-2 and anti-CD3/CD28 beads prior to transduction. Following mononuclear cell (MNC) isolation from a unit of whole blood (2–5 × 10^8 MNC), MNCs were transferred to a 3-liter LifeCell culture bag containing 1 liter X-VIVO-15 culture medium with 10% FFP, 1,000 units IL-2/ml, and anti-CD3/CD28 beads at a ratio of 3 beads/cell. Cells were incubated overnight at 37 °C, 5% CO_2.

Initial process development studies yielded sufficient numbers of transduced, expanded, GCV-sensitive lymphocytes [18]. Engineered lymphocytes, however, demonstrated significantly impaired function, evidenced by impaired phytohemagglutinin (PHA)-induced proliferation and poor function in mixed lymphocyte reactions (MLRs) (fig. 2, 3). These results suggested persistent excessive cell activation, possibly due to prolonged culture with CD3/CD28 beads and IL-2. The initial lymphocyte-engineering process lasted 14 days, with lymphocytes maintained in the presence of CD3/CD28 beads and IL-2 at 1,000 U/ml

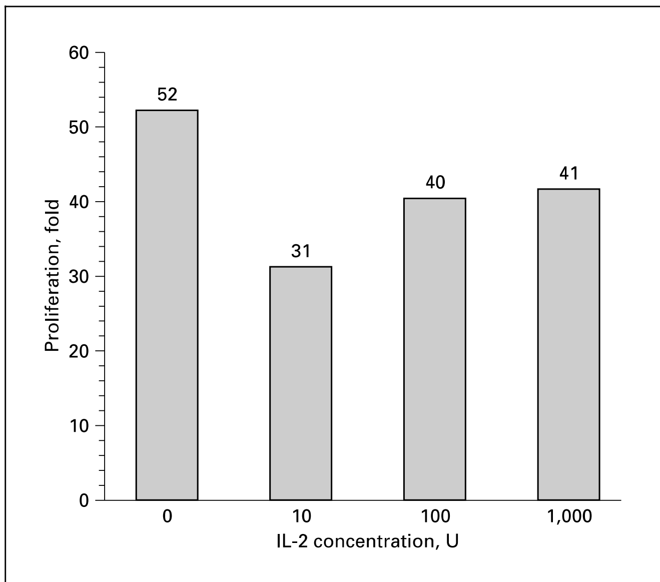

Fig. 4. Effect of IL-2 concentration on expansion of anti-CD3/CD28 activated cells. Donor-derived MNC activated with anti-CD3/CD28 beads in X-VIVO-15 medium were maintained in culture for 12 days in the presence or absence of IL-2. The expansion of cells was determined every 2–3 days, and additional medium added to maintain the cells at a concentration of 0.5 to 2 × 10^6 cells/ml. The overall fold expansion is given for a representative experiment. Similar results were observed in relative expansion in 3 independent experiments.

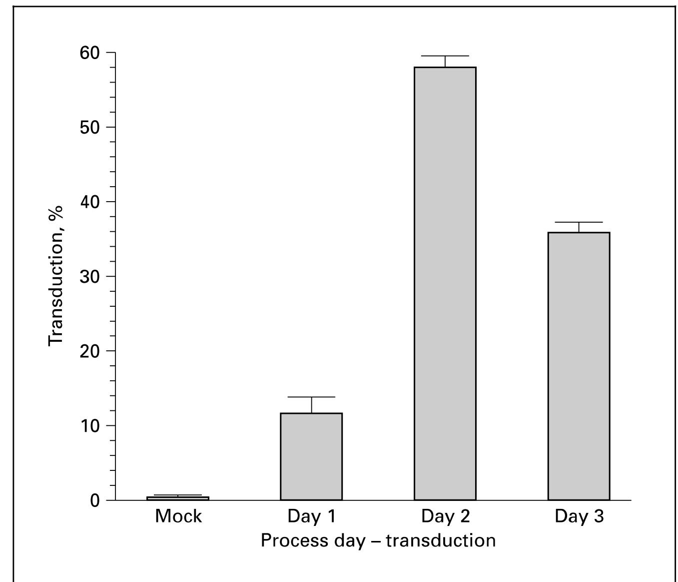

Fig. 5. Transduction frequency based on timing following activation. Donor-derived MNC were activated with anti-CD3/CD28 beads in X-VIVO-15 medium with IL-2 (1,000 U/ml), and 24 h later 2 × 10^5 cells in 400 µl were transduced using MFG-TKiNG supernatant (1.2 ml) in the presence of a final concentration of 8 µg/ml protamine (day 1). Transduction was performed with centrifugation (4,000 *g* for 1 h at 32 °C) and transduction was allowed to continue for 16 h at 37 °C. On day 2 (48 hours after activation) the transduced cells were washed and resuspended in fresh medium, while additional cells (day 2) underwent transduction using an identical procedure; similarly, transduction was performed in the final group (day 3) 72 h after initial activation. The percentage of cells expressing NGFR was determined by flow cytometry 48 h following transduction.

throughout [18]. Small-scale studies testing a range of IL-2 concentrations, however, demonstrated that the presence of IL-2 in the culture medium had little effect on the expansion of the cells subjected to transduction (fig. 4). This suggested that engineered lymphocyte function would be better preserved if IL-2 could be greatly reduced or eliminated from the process without adversely effecting expansion.

Transduction

Modification of T cells for the purposes of both purification of transduced cells (positive selection) as well as the ability to eradicate the cells in vivo (negative selection) requires expression of two genes in a single vector. The MFG-TKiNG retroviral construct (fig. 1) was designed to express tNGFR and HSV-tk using the MFG strategy, which has been shown to provide high efficiency gene transfer and expression [33, 34]. The MFG-TKiNG vector consists of the Moloney murine leukemia virus long terminal repeats, retroviral splice donor/acceptance sites, the herpes simplex virus thymidine kinase (HSV-tk) gene an internal ribosomal entry site and the truncated NGFR cDNA, designed to express both the NGFR and HSV-tk genes from a single bicistronic message, optimizing the potential that the HSV-tk gene will be expressed in cells selected on the basis of NGFR [18].

Transduction conditions, including timing of transduction relative to activation, centrifugation parameters, the effectiveness of transduction with virus produced from PA-317 or PG13 packaging lines, and protein sources were evaluated to develop specifications for increased transduction efficiency and reduced cell loss. Of these parameters, only transduction timing and centrifugation will be discussed here.

Optimal timing of transduction following activation with anti-CD3/CD28 beads was determined by activating MNCs and transducing activated cells in replicates of 3 using MFG-TKiNG supernatant 24, 48 or 72 h after activation. Transduced cell frequency was determined by

flow cytometry, measuring the percentage of NGFR+ cells, with results shown in figure 5. Transduction efficiency was highest when performed 48 h after initial cell activation.

Centrifugation has been used to enhance the physical association of retrovirus and target cells, and the effects on transduction have been shown to increase with both gravitational force and duration of centrifugation [37]. We investigated the effects of centrifugation on transduction frequency and cell viability. The frequency of transduced cells rose with increasing centrifugal force to 4,000 *g* with a minimal effect on the viability of transduced cells (fig. 6).

Based on these data, process specifications were developed which included performing transduction 48 h after initial lymphocyte activation, in protamine (8 μg/ml), with centrifugation at 4,000 *g* for 1 h, followed by overnight incubation at 37 °C.

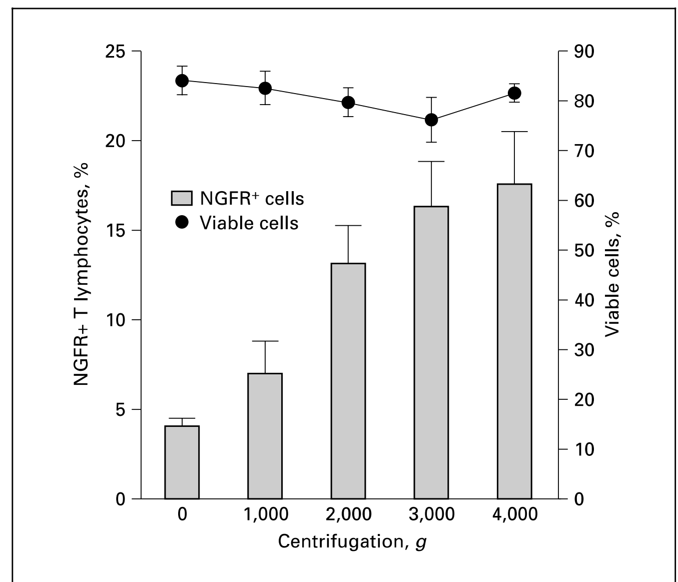

Fig. 6. Effect of centrifugation on transduction frequency and T lymphocyte viability. MNC activated with anti-CD3/CD28 beads were transduced 48 h after activation in triplicate as described in figure 5. Data are pooled from 2 donors (n = 6). Cell viability was measured by acridine orange/propidium iodide darkfield fluorescence microscopy. The frequency of transduced cells was determined by flow cytometry.

Large-Scale Lymphocyte Expansion

The initial process called for expanding cells in culture for 11 days after transduction. Cells were washed and resuspended in IL-2-containing medium, divided into eight 100-ml aliquots in LifeCell bags and cultured at 37 °C, with medium volume doubled after 1, 4, 6, and 8 days. Bags were pooled on culture day 12, and beads removed from cells using the Isolex 300i magnet, with $2.40 \pm 0.98 \times 10^{10}$ (mean ± 1 SD) cells recovered. Cell expansion observed averaged 42.6-fold.

As described above, however, the continued activation of T cells in the presence of IL-2 and anti-CD3/CD28 beads for 12–14 days resulted in cells that were unable to respond to a second stimulus. In addition, the IL-2 was shown to not be required for continued proliferation in vitro. The current protocol has been revised to eliminate exogenous IL-2 from the medium, to remove the anti-CD3/CD28 beads on day 3 of culture, and to end the in vivo expansion period at day 6 rather than day 14. Predictably, cell expansion decreased significantly with the shorter period of ex vivo expansion, averaging 4.6-fold (± 1.6) from day 0 to day 6.

Transduced Lymphocyte Selection

As the purpose of this strategy is to ensure that infused T cells can be eradicated in vivo if severe GVHD is observed, it is essential that transduced cells undergo selection to generate a population of nearly pure transduced cells. We compared two clinical scale immunomagnetic cell selection instruments, the Baxter Isolex 300i and the Miltenyi CliniMACS. Within a single experiment, engineered cells were divided following transduction and expansion and separations performed on using each instrument.

To perform the isolations, 1.0 ml of the anti-NGFR monoclonal antibody 20.4 (2.4 mg) was added to a suspension of 1×10^{10} cells. In the case of the experiments using the Isolex 300i, an unconjugated 20.4 antibody preparation was used, followed by 1.2×10^{10} sheep anti-mouse-IgG paramagnetic beads. For selections performed with the CliniMACS, 1.0 ml of biotinylated 20.4 antibody was used with streptavidin beads (Miltenyi Biotech Inc.). Cell selection with each device was performed in accordance with the manufacturer's specifications.

NGFR+ cell selection using the CliniMACS consistently produced the highest purity cell product, 97.7 ± 2.1% NGFR+ cells, with mean cell yield of 23.5 ± 6.4%. NGFR+ cell selection using the Isolex 300i yielded a product containing 67.5 ± 12.9% NGFR+ cells, with 41.3 ± 13.3% cell recovery (fig. 7). Isolex-selected cells

were incubated overnight, after which bead removal was attempted using the Isolex 300i magnet. Because purity of the engineered lymphocytes is of paramount importance in T-cell suicide gene therapy, to assure that the infused lymphocytes are indeed controllable using GCV pro-drug, the Miltenyi CliniMACS was incorporated into process specifications for transduced cell selection.

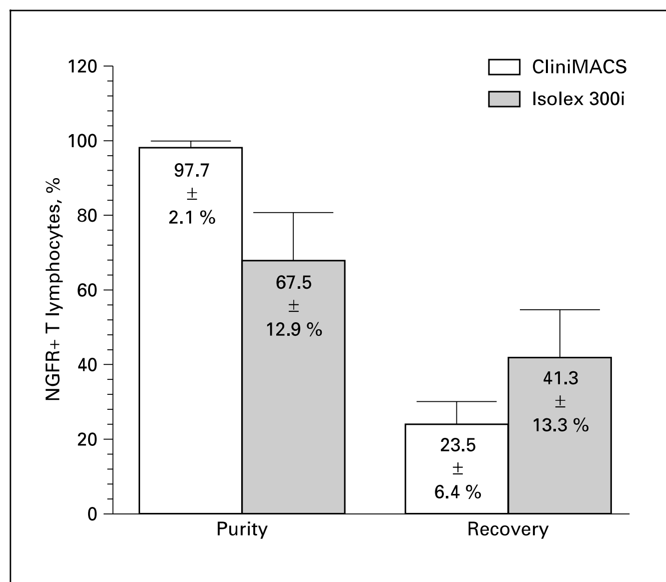

Fig. 7. Immunomagnetic NGFR+ cell selection – instrument comparison, Miltenyi CliniMACS vs. Baxter Isolex 300i. Transduced, expanded lymphocytes were divided into two equal aliquots and processed for positive selection based on NGFR expression. One aliquot was processed using the Baxter Isolex 300i, and the other processed using the Miltenyi CliniMACS unit. Frequency of NGFR+ cells prior to and following selection was determined by flow cytometry. The total yield was determined by calculating the number of NGFR+ cells in a given cell fraction, and comparing this to the number of cells and the proportion of NGFR+ cells after selection.

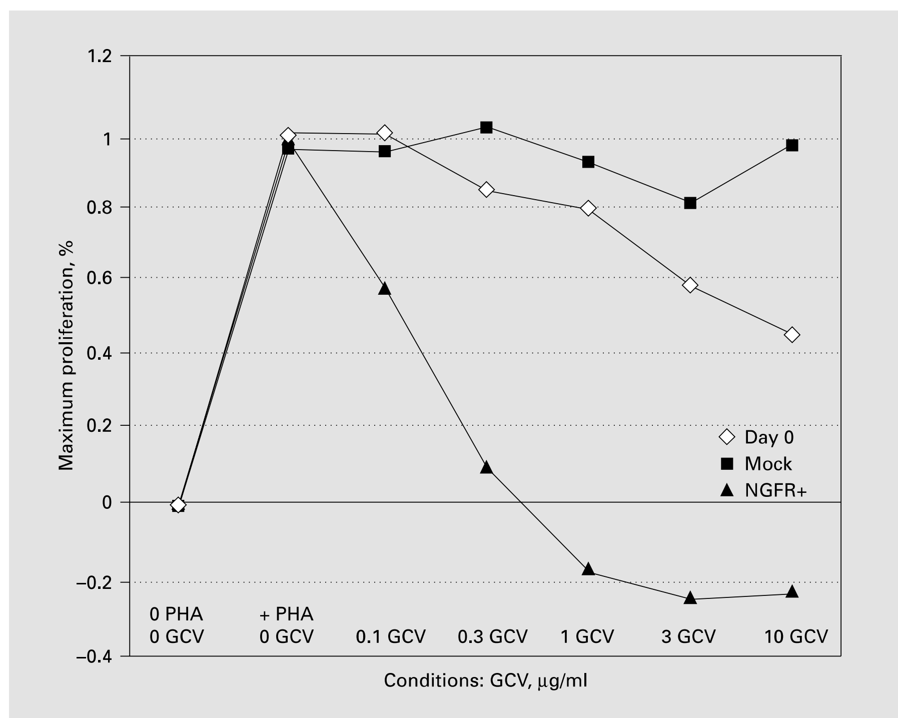

Fig. 8. Ganciclovir sensitivity of NGFR+ cells. Mononuclear cells transduced with the MFG-TKiNG virus on day 2 after activation with anti-CD3/CD28 beads were expanded to day 6, following removal of the beads on the day after transduction. Selection of NGFR+ cells was performed using the CliniMACS device as in figure 7, as was the ability of ganciclovir (GCV) to inhibit proliferation in response to a second stimulatory event (PHA, 10 µg/ml). A total of 10^5 cells from the NGFR+ population, cells from the same donor that were not previously activated (day 0) and cells that were activated and cultured in an identical manner but not transduced (mock) were tested. Increasing concentrations of GCV were used from 0 to 10 µg/ml. Following activation the cells were pulsed with [^{3}H]-thymidine 48 h later for 16 h prior to cell harvesting. Maximal proliferation was defined as [^{3}H]-thymidine incorporation without GCV in the respective groups, and relative proliferation determined. The baseline proliferation was defined as 0%, and in the case of the NGFR+ group proliferation decreased below baseline in the presence of 1, 3 and 10 µg/ml GCV.

Table 3. Specifications for revised lymphocyte engineering process

	Process specifications
Day 0 Cell proliferation	$0.5–2.0 \times 10^9$ total MNC, 5×10^5 cells/ml X-VIVO-15 culture medium Heat-inactivated frozen plasma, 10% Anti-CD3/CD28 beads, 3 beads/cell × 3 days 3-liter LifeCell culture bag
Day 2 Transduction	MFG-TKiNG retroviral vector; 750–1,000 ml Centrifugation, 4,000 *g* × 1 h Protamine, 8 μg/ml
Day 3 Anti-CD3/CD28 bead removal	MaxSep magnetic cell separation device
Days 3–6 Cell expansion	X-VIVO-15 culture medium Heat-inactivated FFP, 10% 3-liter LifeCell culture bag
Day 6 Transduced cell selection	Biotinylated anti-NGFR monoclonal antibody Anti-biotin magnetic particles Miltenyi CliniMACS magnetic cell separation device

Table 4. Cell yields and purity for revised lymphocyte engineering process

	Mean ± 1 SD
Initial cell content	5×10^8 total MNC
Transduction efficiency	22.9 ± 3.9% NGFR+ cells
Cell expansion, days 0–6	4.6 ± 1.6-fold
Cell purity after selection	94.6 ± 3.7% NGFR+ cells
Transduced cell yield	35.3 ± 13.8% overall NGFR+ cell yield

GCV Sensitivity of NGFR-Selected Cells

For these experiments, populations of transduced lymphocytes enriched, using the Miltenyi CliniMACS, for NGFR+ cells were cultured in the presence of varying concentrations of GCV and subjected to activation with PHA. After 48 h, the cells were pulsed with [^{3}H]-thymidine. This appeared to be the optimal time to achieve a proliferative response in cells activated with anti-CD3/CD28 beads for 72 h and maintained in vitro until day 6 (data not shown). Controls included cells from the same donor frozen following mononuclear cell enrichment with Ficoll, and activated, cultured, but non-transduced lymphocytes (fig. 8).

Conclusions

Initial versions of this process generated large numbers of transduced, expanded lymphocytes at appropriately high purity, with demonstrable sensitivity to the GCV pro-drug. Certain aspects of lymphocyte functions were, however, adversely affected due to excessive cellular activation during the process. The current protocol has been modified to more effectively maintain the T-cell proliferative response to an allogeneic stimulus or PHA as a result of three process modifications: (1) eliminating exogenous IL-2 from the process; (2) limiting the ex vivo expansion culture to 7 days, and (3) limiting exposure to anti-CD3/CD28 beads to 3 days.

Revised process specifications for clinical-scale T-lymphocyte transduction, expansion, and selection were developed based on these findings, and are shown in table 3. Engineered lymphocyte yields and purity are summarized in table 4. The process begins with donor lymphocytes obtained from a single unit of whole blood or a 1- to 2-hour apheresis from which 5×10^8 MNCs are isolated, activated with anti-CD3/CD28 beads, and transduced on day 2 using centrifugation (4,000 *g* for 1 h) and 8 μg/ml protamine. On day 3 the cells are washed and anti-CD3/CD28 beads removed. The cells are cultured in X-VIVO 15 with 10% FFP until day 6, when selection is performed using a biotinylated anti-NGFR monoclonal antibody and anti-biotin beads, selecting NGFR+ cells using the Miltenyi CliniMACS.

Transduction efficiency averaged 22.9 ± 3.9% in development runs. Following selection transduced cells were enriched to extremely high purity (94.6 ± 3.7%). Cell expansion from day 0 to 6 averaged 4.6-fold (±1.6), with 35.3 ± 13.8% overall yield of NGFR+ cells. The NGFR+ fraction was tested for sensitivity to GCV by determining inhibition of the proliferative response on day 2 following PHA stimulation. Engineered lymphocytes were demonstrably GCV-sensitive. GCV concentrations of 1 μg/ml resulted in 80% inhibition of PHA-induced proliferation, confirming that levels of GCV attainable in serum arrest engineered T cell expansion.

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Alternative Concepts of Suicide Gene Therapy for Graft-versus-Host Disease after Adoptive Immunotherapy

Christof M. Kramm

Department of Pediatric Hematology, Oncology, and Immunology, University Hospital Düsseldorf, Düsseldorf, Germany

Key Words
Suicide gene · Gene therapy · Graft-versus-host disease · Donor lymphocytes · Leukemia

Abstract
T-cell suicide gene therapy represents a promising novel treatment strategy for graft-versus-host disease following adoptive immunotherapy after allogeneic hematopoietic stem cell transplantation. The clinical efficiency of this approach is still hampered by several obstacles including induction of alloresponses due to the use of immunogenic suicide and selection genes, genetic inactivation of suicide genes, and functional immunological impairment after retroviral transduction with extensive in vitro stimulation. New concepts as possible solutions to these limitations are discussed.

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Introduction

Adoptive immunotherapy employing donor lymphocyte infusions (DLI) represents a potent instrument to treat relapses of chronic myeloic leukemia after allogeneic hematopoietic stem cell transplantation (alloHSCT). Here, remission is re-achieved in up to 70% of treated patients [Kolb et al., 1995] demonstrating a strong graft-versus-leukemia (GvL) effect. For post-transplantation relapses of acute leukemia, treatment results with DLI are less impressive (acute myeloblastic leukemia: 25% complete response; acute lymphoblastic leukemia: <15% complete response) [Kolb et al., 1995; Porter et al., 1996; Collins et al., 2000], but acute leukemia patients may benefit when DLI are started at the early stage of increasing mixed chimerism indicating the imminent hematological relapse [Bader et al., 1999; Beck et al., 2002].

Application of DLI on a general basis after alloHSCT is hampered by the high risk of graft-versus-host disease (GvHD). Approximately 50% of the patients suffer from clinically significant GvHD after DLI [Kolb et al., 1995; Collins et al., 1997]. The incidence and degree of GvHD appear to be correlated with the amount of applied donor lymphocytes.

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Fax + 41 61 306 12 34
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Christof M. Kramm, MD
Department of Pediatric Hematology, Oncology, and Immunology
University Hospital Düsseldorf, Heinrich Heine University, Moorenstrasse 5
DE–40225 Düsseldorf (Germany)
Tel. +49 211 8117662, Fax +49 211 8117634, E-Mail kramm@uni-duesseldorf.de

T-Cell Suicide Gene Therapy

Transfer of suicide genes represents the most recent strategy to control the risk of GvHD after DLI. Suicide genes code for enzymes of pro- or eukaryontic origin which are capable of activating an inert substance ('prodrug') into highly cytotoxic metabolites. Cytotoxic metabolites are only generated in genetically modified ('transduced') cells and will predominantly kill these cells while sparing non-transduced ones.

To date, only the herpes simplex virus type 1 gene for thymidine kinase *(HSV-tk)* has been employed in clinical trials as a suicide gene. *HSV-tk* initializes the intracellular cytotoxic activation of the nucleoside analogons acyclovir and ganciclovir (GCV). Only GCV is used for suicide gene therapy purposes since HSV thymidine kinase displays a significantly higher affinity for this prodrug than for acyclovir [Aghi et al., 2000]. Using highly specialized transduction and selection protocols and procedures, more than 95% of donor lymphocytes can be transduced with the *HSV-tk* gene in vitro by retroviral gene transfer. Application of these genetically modified donor lymphocytes provides an efficient tool to abrogate GvHD since *HSV-tk*-positive GvHD effector cells can be eliminated by GCV treatment. Control of GvHD by T-cell suicide gene therapy has indeed been shown in patients after alloHSCT [Bordignon et al., 1995; Bonini et al., 1997; Verzeletti et al., 1998; Tiberghien et al., 2001].

Immunological Inactivation of Genetically Modified Donor Lymphocytes

However, not all patients appeared to benefit from T-cell suicide gene therapy and suffered from GvHD despite GCV treatment. One reason may be that genetically modified T cells are inactivated by immune responses to non-human gene products such as the HSV-TK protein [Verzeletti et al., 1998]. Tiberghien et al. [2001] attempted to overcome this unwanted immune response in their clinical trial by applying *HSV-tk* transduced donor lymphocytes not in case of relapse, but in parallel to the transplantation of T-cell-depleted grafts. By this procedure, an immunological tolerance towards the *HSV-tk* gene product should be induced. At least a partial prevention of immunological inactivation of genetically modified donor lymphocytes was supposed to have occurred since observed GvHD rates were only slightly lower than those found after application of unmodified donor lymphocytes. However, despite the possible induction of an immune tolerance towards the suicide gene, one patient still suffered from GvHD which could not be terminated by GCV.

An interesting modification of this add-back principle was performed by Litvinova et al. [2002]. In an animal model, genetically modified donor lymphocytes were applied at defined time points after alloHSCT together with a short treatment cycle of GCV to remove activated donor T cells. With this approach, the GvHD incidence was reduced, but a marked GvL effect was still found.

New Suicide and Selection Marker Genes with Lower Immunogenic Potential

Less immunogenic suicide and selection marker genes, preferably of human origin, may also reduce the immunological inactivation of genetically modified donor lymphocytes. Truncated versions or splice variants of the human genes for low affinity nerve growth factor receptor (LNGFR) and CD34 [Fehse et al., 1998, 2002] are already used instead of the highly immunogenic bacterial neomycin resistance gene for selection of transduced donor lymphocytes. Especially appealing seems a fusion gene construct of *CD34* and *HSV-t*k which provides both selection and suicide gene properties [Fehse et al., 2002].

The development of an inducible fusion gene employing the human *fas* gene as cytotoxic component may also contribute to further reduction of immunogenicity. This new suicide gene codes for a chimeric protein which contains the membrane-anchored intracellular domain of Fas fused to the FK506-binding protein FKBP12. Addition of the dimerizing prodrug AP1903 induces Fas cross-linking with subsequent triggering of an apoptotic death signal. This suicide gene therapy system has been shown in vitro to confer a reliable cytotoxicity to human T cells. In contrast to the *HSV-tk*/GCV system, only short prodrug exposures were sufficient to induce a rapid T-cell killing, independently of cellular proliferation [Thomis et al., 2001].

An equally interesting new approach is offered by transfer of the human gene for CD20 and application of the chimeric anti-CD20 antibody rituximab as cytotoxic principle [Introna et al., 2000, 2002]. Rituximab binds to the B-cell-specific CD20 surface antigen and can induce lysis of CD20-positive cells in the presence of complement. Thus, transduction of T cells with *CD20* provides both a selection marker and a suicide gene. One pitfall of this approach may be additional depletion of circulating B cells by rituximab although rituximab treatment of non-

Hodgkin lymphoma has already been shown to be relatively safe without major side effects [McLaughlin et al., 1998].

Other human-derived prodrug-activating systems like the human folylpolyglutamate synthetase (hfpgs)/methotrexate (MTX), the deoxycytidine kinase (dCK)/cytosine arabinoside (ara-c), or the carboxylesterase (CE)/irinothecan (CPT-11) systems are also interesting candidates for further exploration since they promise a low potential of immunogenicity [Hapke et al., 1996; Kojima et al., 1998; Aghi et al., 1999]. However, these systems represent modified suicide gene therapy strategies since they do not activate non-toxic prodrugs but enhance already potent chemotherapeutic agents. Thus, these systems may provide the basis for a dose reduction of the corresponding chemotherapeutic agents. Future studies still have to clarify if the dose reduction of chemotherapeutic agents due to application of suicide gene therapy systems may be sufficient to circumvent myelotoxicity in the post-transplantation course.

However, MTX is already used as an immunosuppressant to prevent early GvHD after alloHSCT. Transfer of the suicide gene *hfpgs* may enhance cytotoxicity in transduced T cells since polyglutamylation of MTX will be induced, thereby increasing its intracellular retention and its overall inhibitory effect on nucleic acid synthesis. Thus, the application of MTX in case of severe GvHD may not only kill transduced donor lymphocytes, but may also provide additional inhibitory activity on non-transduced, but activated T cells. Thus, the effect on GvHD may not be as selective as by the *HSV-tk*/GCV system, but it may be more potent. A modification of this approach has been reported by Liu et al. [2002]. Here, the murine-reduced folate carrier *(mRFC)* gene was employed to enhance sensitivity of hematopoietic cells to MTX. Transfer of this gene increases the cellular uptake of MTX. In a murine bone marrow transplantation model, MTX treatment resulted in a significant decrease of hematopoietic cells transduced with *mRFC*. Since *mRFC* is a murine gene with potential immunogenicity, future exploration of this system should focus on the human analogue *RFC1* [Moscow et al., 1995].

Rapid killing of transduced cells independently of cellular proliferation was also observed with the rabbit cytochrome P_{450} isotype 4B1 (cyp4B1)/4-ipomeanol (4-IPO) system in a rodent brain tumor model. Here, cell killing was induced more rapidly than by the *HSV-tk*/GCV system [Frank et al., 2002]. Preliminary studies have already confirmed a reliable induction of similar cytotoxicity by the *cyp4B1*/4-IPO system in T cells [Kramm, Steffens, Kluemper, unpubl. results]. However, the relevance of any immunogenicity due to expression of rabbit proteins still has to be cleared.

Table 1 summarizes data of possible suicide gene therapy systems [Aghi et al., 2000] with special respect to their feasibility for T-cell suicide gene therapy. Most of the systems appear not to be eligible for this purpose since they are of bacterial or yeast origin and may, therefore, yield a highly immunogenic potential. Furthermore, only less than 50% of these systems have actually been tested in T cells. Interestingly, the *Escherichia coli* cytosine deaminase *(cd)*/5-fluocytosine (5FC) system which represents the only other suicide gene therapy system which has been used besides the *HSV-tk*/GCV approach in clinical cancer trials was shown to induce no significant cytotoxicity in T cells [Hiller et al., 2000].

Data regarding the so-called bystander effect which describes the phenomenon that non-transduced cells in close proximity to transduced cells can be killed upon prodrug treatment by transfer of cytotoxic metabolites have also been included into table 1. For tumor treatment, the bystander effect represents one of the cornerstones of therapeutic efficiency of suicide gene therapy systems. For T-cell suicide gene therapy, the bystander effect appears to be less important since transduced and non-transduced cells are usually not as close to each other as they may be within a tumor environment. However, regarding T-cell infiltration of solid organs like the liver induction of a bystander effect upon prodrug treatment may be also disadvantageous by enhancing organ toxicity.

Reduced Immunological Competence of Genetically Modified Donor Lymphocytes

Besides the immunological inactivation due to an alloreaction against non-human suicide or other vector gene products, there is an increasing number of reports which demonstrate a markedly reduced immune competence of genetically modified cells when compared to their unmodified counterparts [Tiberghien 2001; Sauce et al., 2002; Duarte et al., 2002]. There is no doubt that genetically modified T cells maintain certain functions essential for alloreactivity [Di Ianni et al., 2000]. However, in vitro cell culture, retroviral transduction, and selection procedures appear to have a substantial negative impact on the overall immunological functionality as shown for the anti-Epstein-Barr virus (EBV) potential of genetically modified T cells [Sauce et al., 2002]. This observation provides

Table 1. Feasibility of different prodrug-activating systems for T-cell suicide gene therapy

Suicide gene therapy system	Origin	Expected immunogenicity	Expected myelotoxicity	Proliferation dependence of cytotoxicity	Bystander cell death induction by diffusion	Induction of cell death in T cells
HSV-tk/GCV	HSV type 1	High	Possible	Yes	Only by cell-to-cell contact	Yes
fas-FKBP12/AP1903	Human	Low	Probably not	No	Unknown	Yes
CD20/rituximab	Human	Low	Highly cytotoxic for B cells	No	Unknown	Yes
hfpgs/MTX	Human	Low	Possible	Probably not	No	Unknown
mRFC/MTX	Human or mouse	Low or moderate	Possible	Probably not	Probably not	Yes (in mice)
dCK/Ara-c	Human	Low	Possible	No	No	Unknown
ce/CPT-11	Human or rabbit	Low or moderate	Possible	Unknown	Yes	Unknown
cyp2B1/CPA	Rat	Moderate	Possible	No	Yes	Unknown
cyp4B1/4-IPO	Rabbit	Moderate	Probably not	Probably not	No	Yes
Nitroreductase/CB1954	*E. coli*	High	Unknown	Yes	Yes	Yes
cd/5FC	*E. coli* or yeast	High	Probably not	No	Yes	No
deoD/MeP	*E. coli*	High	Unknown	Unknown	Yes	Unknown
gpt/6-TX	*E. coli*	High	Probably not	Unknown	Only by cell-to-cell contact	Unknown

cyp2B1/CPA = Cytochrome P_{450} isotype 2B1/cyclophosphamide; *deoD*/MeP = purine nucleoside phopsphorylase/6-methyl-purine-2′-deoxynucleoside; *gpt*/6-TX = guanine phosphoribosyl transferase/6-thioxanthine.

a sufficient explanation for the occurrence of an EBV-associated lymphoproliferative disease after alloHSCT despite the application of genetically modified donor lymphocytes which – in their native form – usually represent a potent therapeutic tool for this disease [Tiberghien et al., 2001].

Although there have been improvements of the immunological competence due to changes in conditions for cell culture and selection [Sauce et al., 2002], the use of retroviral vectors for gene transfer still appears to represent the limiting step for further positive developments. Retroviral gene transfer can only occur during cellular proliferation, but the necessary in vitro stimulation of T cells to proliferate is accompanied by functional impairment [Duarte et al., 2002]. The use of lentiviral vectors which can also transduce non-proliferative cells may offer a solution in this respect. One study already showed a significantly improved preservation of T-cell functionality after lentiviral *HSV-tk* gene transfer [Cazzaniga et al., 2002]. Despite several striking advantages including a markedly enhanced gene transfer efficiency, to date, there is still no authorization to use lentiviral vectors in clinical trials. The fact that these vectors are derived from human immunodeficiency virus may be one of the main reasons for this.

Potential Risk of Malignant Transformation

Most recently, the occurrence of T-cell leukemia-like proliferative disease in 2 patients who had undergone retrovirus-mediated gene therapy for X-linked SCID has started a controversy about the usage of integrating vectors like retroviral and lentiviral vectors. In addition, the use of the LNGFR selection marker which is employed for clinical T-cell suicide gene therapy was also linked in a murine model of hematopoietic stem cell gene therapy to the induction of acute leukemia [Li et al., 2002].

These current developments have shocked the entire scientific community since vector-mediated genomic integration of transgenes has long been regarded to occur on a purely random basis with a minimalized risk for interference with host's genetic elements crucial for prevention of malignant transformation. Extensive present and fu-

Table 2. Limitations of present T-cell suicide gene therapy and possible future solutions

	Limitations	Clinical impact	Possible solutions
Suicide gene therapy system	Immunogenicity	Immunological inactivation with reduced GvL effect	Use of human-derived suicide genes DLI as add-back to T-cell-depleted graft
	Loss of genetic material	Non-functional suicide gene product with GCV resistance and no therapeutic efficiency against GvHD	Modification of suicide gene sequence Combination of different suicide genes
	Pharmacological resistance	GCV resistance without therapeutic efficiency against GvHD	Combination of different suicide genes
Selection marker	Immunogenicity	Immunological inactivation with reduced GvL effect	Use of human-derived selection markers
	Possible risk of malignant transformation by LNGFR	Acute leukemia?	Use of other human-derived selection markers, e.g. truncated *CD34* gene
Retroviral vector	Need for in vitro T-cell stimulation	Impairment of immunological functions with reduced GvL effect and reduced protection against infectious agents	Use of lentiviral vectors
Retroviral vector (+ lentiviral + foamy virus vectors)	Risk of malignant transformation by genomic integration	Acute leukemia	Development of vectors with stable episomal gene expression or site-specific genomic integration

ture investigations have now to clarify the actual malignant potency of retroviral and lentiviral gene therapy. Vectors with a defined safe integration site or stable episomal gene expression may represent an attractive alternative for future clinical gene therapy. However, they still have to be further explored [Hiller et al., 2000] or even developed.

Genetically Induced and Pharmacological Resistance to GCV Treatment

There have been also hints in previous clinical studies for a resistance of genetically modified donor lymphocytes to GCV treatment [Bonini and Bordignon 1997]. Garin et al. [2001] demonstrated that this may reflect in some cases the presence of a non-functional *HSV-tk* splice variant. This splice variant which is estimated to occur at a frequency of 10–20% is characterized by a deletion of 227 base pair fragment resulting in expression of a truncated HSV-TK protein not capable of activating GCV. By modifying the *HSV-tk* gene sequence, generation of truncated HSV-TK proteins has been abolished [Chalmers et al., 2001].

Besides the genetic inactivation of the *HSV-tk* gene, there may also be true pharmacological resistance mechanisms which may hamper an efficient GCV treatment of *HSV-tk*-expressing cells. Two GCV resistance mechanisms involving a cellular enzyme for DNA repair and a multidrug resistance protein have been recently described [Tomicic et al., 2002; Adachi et al., 2002]. As in most modern multimodal treatment protocols for malignant diseases, the combination of different cytotoxic agents, i.e. different suicide gene therapy systems, may help to prevent and/or overcome pharmacological resistance mechanisms. Combination of the *HSV-tk*/GCV and the *cd*/5FC systems has already been shown to induce a therapeutic synergism in the treatment of experimental brain tumors [Aghi et al., 1998].

Outlook (table 2)

Future concepts in T-cell suicide gene therapy have to rely on suicide and selection genes with low immunogenicity. Low immunogenicity may be achieved by use of human-derived transgenes, such as *fas-FKBP12* or *CD20* as suicide genes or the *CD34* splice variant gene as selec-

tion marker. Induction of an immune tolerance towards non-human transgenes, e.g. by application of genetically modified donor lymphocytes as add-backs to T-cell-depleted grafts, may be an alternative. In general, the combination of different suicide gene therapy systems appears to be advantageous to enhance the overall therapeutic efficiency and to prevent resistance development. Retroviral vectors should be replaced by vectors which can transduce T cells without the need of cellular proliferation. The avoidance of extensive in vitro stimulation will help to reduce the loss of immunological competence important for GvL effect and protection against infectious agents. To date, lentiviral vectors appear to offer the most suitable features for T-cell suicide gene therapy. However, lentiviral vectors still need to be authorized for clinical trials, and their risk of inducing malignant transformation has to be carefully assessed.

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Adoptive T-Cell Therapy for EBV-Associated Post-Transplant Lymphoproliferative Disease

Catherine M. Bollard   Barbara Savoldo   Cliona M. Rooney   Helen E. Heslop

Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Tex., USA

Key Words
Transplantation · Epstein-Barr virus · Adoptive immunotherapy · Post-transplant lymphoproliferative disease

Abstract
Increased understanding of the mechanisms by which T lymphocytes recognize virus and tumor-specific antigens has fueled the use of adoptive immunotherapy for viral and malignant diseases. An ideal candidate for such treatment is Epstein-Barr virus (EBV). EBV-associated post-transplant lymphoproliferative disorder (PTLD) is a serious complication post-solid organ transplant (SOT) or hematopoietic stem cell transplant (HSCT). The disease is essentially the result of suppression of cytotoxic T-cell function and despite various treatment strategies the course may still be fulminant and lethal. Therefore, an adoptive immunotherapeutic approach using ex vivo derived EBV-specific CTL offers a promising solution not only for the treatment but also as prophylaxis for PTLD. The infusion of EBV-CTL has been demonstrated to be safe and effective in allogeneic HSCT recipients and their use post-SOT is being evaluated.

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Introduction

Epstein-Barr virus associated post-transplant lymphoproliferative disorder (PTLD) is a potentially life-threatening consequence of immune suppression post marrow and solid organ transplantation. Persistence and reactivation of EBV infection within the cells of the immune system is a unique characteristic of gamma herpes viruses and is fundamental to the pathogenesis of this EBV-associated disease. PTLD is heterogeneous in its presentation ranging from polyclonal lymphoproliferations to malignant lymphoma. In addition, the clinical presentation and course as well as treatment options differ when PTLD develops post organ transplant versus marrow transplant. Although there are a number of therapeutic options including chemotherapy and treatment with monoclonal antibodies, PTLD can still have a rapid and lethal course. However, a promising approach for the treatment and prevention of this devastating illness is the application of adoptive immunotherapy such as the use of ex vivo derived EBV-specific cytotoxic T lymphocytes (CTL) to restore the deficient CTL response.

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Dr. Helen E. Heslop
Center for Cell and Gene Therapy
1102 Bates St, Suite 1140
Houston, TX 77030 (USA)
Tel. +1 832 824 4662, Fax +1 832 825 4668, E-Mail hheslop@bcm.tmc.edu

Epstein-Barr Virus Infection

EBV is an enveloped herpesvirus with a 172-kb double-stranded DNA genome [1]. In the immunocompetent host, EBV infection results in a mild self-limiting illness [2]. Over 95% of the adult population worldwide are seropositive for EBV primarily after developing the infection during childhood [3]. Like other herpes viruses, EBV is then able to maintain a latent infection with the virus genome retained in the host cells without production of infectious virions. EBV targets oral epithelial cells and B cells. The CD21 receptor of the B lymphocyte allows the EBV to enter the cell and establish latent infection in vivo with the expression of latency-associated transforming proteins [4]. There are three types of EBV latency defined by the number and type of latent protein expressed on the host's B cells. During acute infectious mononucleosis, between 1 in 100 and 1 in 1,000 circulating B cells in the peripheral blood are infected with EBV and express a type 3 latency pattern of transcription [5]. Type 3 latency involves the expression of EBV nuclear antigen (EBNA)-1, -2, -3A, -3B, -3C, the leader protein (LP), latent membrane protein (LMP)-1, -2A, -2B and the cytosolic protein RK-BARFO (the product of the *Bam*H1 open reading frame) [6]. In addition, abundantly expressed small non-polyadenylated RNAs termed EBV early RNA (EBER)-1 and -2 are transcribed but not translated [7]. The expression of the 9 viral antigens and the presence of cell adhesion and co-stimulatory molecules make these B cells highly immunogenic and in the immunocompetent host, susceptible to immune mediated killing by EBV-specific cytotoxic T cells [6]. In addition, in vitro type 3 latency is demonstrated by the establishment of immortalized EBV-transformed lymphoblastoid B cell lines (LCL). In the immunocompetent host, once the type 3 latency-expressing B cells are eliminated by the cellular immune response, around 1 in 10^6 infected B cells persist which express a more restricted pattern of EBV genes. This viral gene expression in resting infected B cells in normal seropositive individuals, is limited to the immuno-subdominant LMP-2A antigen, BARFO, EBNA-1 which possess gly-ala repeat sequences that inhibit HLA class I antigen processing, and EBERs [4, 5]. This expression of a minimal subset of genes, which are weak targets for CTL activity, therefore allows infected cells to evade the immune system and maintain a low level persistent infection [8].

Pathogenesis of EBV-Driven Lymphoproliferation (PTLD)

In seropositive individuals, EBV-infected cells may undergo lytic replication usually with a transient expression of the full panel of type 3 latent antigens, followed by immune recognition and elimination by EBV-specific CTL. In contrast, type 1 and type 2 latency express only a limited array of EBV antigens as seen in nasopharyngeal carcinoma, Hodgkin disease and Burkitt's lymphoma [9–11].

An ongoing balance exists in normal seropositives between the EBV viral load and the immune defense mechanisms. However, following transplantation where cytotoxic T-cell numbers and/or activity are drastically suppressed, the EBV-infected B cells expressing a type 3 latency are able to proliferate unchecked [12]. This leads to accumulation of EBV-infected B cells in the body and enhanced viral replication as demonstrated by elevated levels of EBV DNA detected in the peripheral blood by polymerase chain reaction (PCR) [13–16]. These changes reflect the loss of CTL activity and in many patients, uncontrolled EBV-driven B-cell lymphoproliferation occurs leading to immunoblastic lymphoma.

EBV-Driven Lymphoproliferation in Stem Cell Transplantation

Incidence and Risk Factors

The differences in the incidences between PTLD developing after solid organ versus stem cell transplants are highlighted in table 1. The reported incidence of PTLD post-hematopoietic stem cell transplant (HSCT) ranges from 1 to 25% of transplant recipients [17]. The highest frequencies are seen in patients receiving HSCT from mismatched family donors or unrelated donors, particularly if the marrow was depleted of T cells to prevent graft-versus-host disease [18–23]. The post-transplant immunosuppression regimen employed is also an important risk factor for PTLD. The type and cumulative dose of potent T-cell-immunosuppressive agents such as anti-CD3 antibody, cyclosporin, FK506 and antithymocyte globulin [24, 25] play an important role in determining risk for EBV-associated PTLD. In addition, bone marrow transplant recipients with underlying immunodeficiencies such as Wiskott-Aldrich syndrome represent an independent risk group for PTLD [26].

Table 1. Incidence of PTLD in allorecipients

	Type of transplant	Incidence of EBV-PTLD
Hematopoietic stem cell	Mismatched	1%
	Mismatched + T-cell depletion	1–8%
	Unrelated	1.5%
	Unrelated + T-cell depletion	5–29% [33, 35, 48–50]
	Unrelated + Campath depletion	1.3%
Solid organ	Kidney	1–2% [56, 57]
	Heart	5–10% [58, 59]
	Liver	2–10% [60, 61]
	Small bowel	20% [60]
	Lung and heart-lung	9–19% [58, 59]

Pathology and Clinical Presentation

Ablation of the recipient's bone marrow before transplantation and reconstitution of the recipient's immune system with donor lymphocytes means that PTLD is usually of donor B-cell origin [19]. In the bone marrow transplant setting, PTLD usually presents early post-transplant and is rapidly fatal [19]. EBV-associated PTLD that arises in allogeneic stem cell recipients are usually classified as immunoblastic lymphomas [27]. The majority of the tumor cells express a type 3 latency pattern of gene expression and are phenotypically identical to LCL derived in vitro [27, 28]. The diagnosis of PTLD post-HSCT is usually established within the first 3 months following transplant with the range being 3–12 months when profound deficiencies of EBV-specific CTL are observed [20]. In these patients with early-onset PTLD, the EBV-infected cells are donor-derived. There is a small subset of HSCT patients who develop 'late-onset' PTLD up to 4 years post-transplant [29]. Generally, these patients require HSCT for underlying immunodeficiency syndromes and incomplete engraftment post-transplant. In contrast to the early-onset group, the EBV-infected cells were of host origin.

The clinical presentation of PTLD post-HSCT may be a generalized systemic illness not unlike infectious mononucleosis with prominent B symptoms (fevers, sweats, anorexia) and rapid enlargement of the tonsils and cervical nodes [30]. In highly immunosuppressed patients, they may present with fulminant disease with diffusely infiltrative multiorgan involvement difficult to distinguish from sepsis or graft-versus-host disease [31]. Retrospective analyses of several case reports and studies reported that PTLD emerged as an incidental finding, discovered at autopsy, in nearly 20% of patients [32–34]. PTLD can therefore be very variable in its presentation and not always easily recognizable. Prompt diagnosis and intervention are likely to improve outcome, which is why many bone marrow transplant centers monitor patients' EBV-DNA levels post-transplant [15, 35–38]. Using quantitative PCR, EBV-DNA levels >4,000 copies/µg DNA are highly predictive for the development of EBV lymphoma and therefore serve as a guide to commence treatment [39].

Therapeutic Management

Therapeutic approaches to PTLD post-HSCT such as reduction of immunosuppression and the use of the antiviral agents acyclovir and ganciclovir have not substantially improved the poor prognosis of PTLD in this setting [34, 40]. Post-HSCT, the withdrawal of immunosuppressive therapy is usually ineffective as the developing donor-derived immune system cannot provide sufficient immune recovery to eradicate EBV-infected B cells in bone marrow transplant patients. Chemotherapy and localized radiotherapy have been used to treat PTLD post-HSCT, but mortality is high secondary to significant cytotoxicity [41]. The use of monoclonal anti-B cell antibodies has also been investigated for the treatment of this disease. A mixture of anti-CD21 and anti-CD24 antibodies was found to be effective in a European multicenter trial which included HSCT recipients with PTLD. Twenty-seven patients post-HSCT were treated with an overall survival rate of 46% at a median follow-up of 61 months [42, 43]. The toxicity was mild, but these antibodies are not available clinically. Our group and others have investigated the feasibility of treating PTLD with the humanized murine anti-CD20 antibody Rituximab (Rituxan; IDEC Pharmaceuticals, San Diego, Calif. and Genentech, Inc., San

Francisco, Calif., USA) [44–47]. Although follow-up is relatively short and the patient numbers in each series is relatively small, the overall response rates ranged from 69 to 100% and therefore represents a promising strategy for PTLD post-HSCT. In addition, the reported toxicity was mild, however the long-term sequelae are as yet unknown – in particular the risk of selecting CD20-negative tumors.

Adoptive Immunotherapy Approach in HSCT Recipients

It has been possible to restore EBV-specific immunocompetence and therefore control EBV LPD post-HSCT by administering unselected populations of donor lymphocytes to transplant recipients [48, 49]. However, the utility of such therapy is limited by potentially fatal complications that arise from alloreactive T cells also present in the lymphocyte infusion [48, 49]. To overcome this obstacle, several groups have investigated the feasibility of generating donor-derived EBV-specific CTL [50–53]. Our group generated EBV-specific T-cell lines from donor lymphocytes and used them as prophylaxis and treatment for EBV-induced lymphoma in patients post-HSCT [50]. CTL were generated by initiating an LCL line by infecting donor peripheral blood mononuclear cells with a laboratory strain of EBV. These LCL which, as mentioned previously, express a type 3 latency, were irradiated and then used as antigen-presenting cells to stimulate and expand EBV-specific CTL from the donor lymphocyte population. Over a 7-year period, 56 patients who received a T-cell-depleted HSCT were given EBV-CTL prophylactically. The patients ranged in age from 9 months to 20 years and CTL were administered at a median of 88 days post-HSCT. The first 26 patients enrolled on to the study received CTL which were genetically modified with the neomycin resistance gene. None of the 56 patients who received the EBV-CTL developed PTLD compared with an incidence of 11.5% in a comparable group who did not receive CTL [22]. Using conventional PCR and real-time PCR, the marker gene was identified in the peripheral blood at least 69 months post-CTL [54]. Although toxicity was low in this prophylaxis group, inflammatory responses were seen in patients who had incipient disease. Three patients who declined or were ineligible for our prophylaxis study were treated for established EBV lymphoma. The EBV-specific CTL therapy induced a remission for 2 patients although in 1 case dramatic inflammation occurred at sites of disease after CTL administration. The patient who failed treatment was found to have an escape mutant in her EBV lymphoma cells. Therefore, although the donor EBV-CTL line recognized two immunodominant HLA-11-restricted epitopes in EBNA-3B, the patient's tumor cells had a mutation in the EBNA-3B epitope thereby rendering the tumor resistant to the donor CTL [55]. Although polyclonal CTL lines were used, it is possible for escape mutants to arise in this setting. Therefore, the risk of tumor escape mutants remains a concern as it does with the use of monoclonal antibody therapies. In addition, the infusion of CTL to patients with incipient or established disease warrants caution secondary to the inflammatory response at disease sites. However, adoptively transferred EBV-CTL do persist long term and can prevent as well as effectively treat EBV driven PTLD.

PTLD in Solid Organ Transplant (SOT) Recipients

Incidence and Risk Factors

In SOT recipients, the incidence of PTLD varies according to the type of graft, being lower after renal, heart or liver transplant (1–10%) and higher after lung or small bowel transplant (10–30%) [56–61]. These differences depend on the degree and duration of the immunosuppressive treatment employed to prevent graft rejection. The other major risk factor for the development of PTLD is the occurrence of a primary EBV infection after the transplant [25]. Therefore, the pediatric populations who are recipients of solid organ grafts are particularly susceptible to PTLD, because children are more frequently EBV-seronegative at the time of transplant. EBV infection of the recipient may result from different sources including latent EBV infection of donor or host lymphocytes, blood transfusions and the graft itself [34].

Clinical Presentation and Pathology

Although seroconversion after EBV infection may be asymptomatic, up to 50% of patients present with fever, lymphadenopathy, rash and diarrhea along with a high EBV-DNA viral load at initial infection. Symptomatic primary infection is frequently followed by PTLD. In EBV-seropositive SOT recipients, PTLD can present as single or localized forms, with regional node involvement. Symptoms are related to the anatomic site involved. EBV-driven lymphoma can localize near the transplanted liver or kidney. PTLD can present as diffuse disease, with multiorgan involvement, including CNS [62, 63].

Different histologic subtypes have been described, ranging from benign hyperplasia, generally polyclonal, to monomorphic and mononclonal forms. According to the interval of development of disease after transplant, PTLD can be divided into 'early' and 'late' lymphoma. Their development closely related to the intensity of immunosuppression given in the first period post-transplant, since the former develops within the first year of transplant and is invariably associated with EBV. In contrast, the 'late' form develops 2–5 years or later after the transplantation and their pathogenesis is probably multifactorial with a significant number being EBV-negative [64–66]. These late-onset lymphomas also have a much poorer prognosis and require more aggressive therapies.

Therapeutic Management

Reduction or withdrawal of immunosuppression, aimed at restoring the ability to control the proliferation of the infected B cells, is suggested as first-line treatment of PTLD post-SOT [67]. Regression of PTLD has been reported after suspension of immunosuppressive drugs, though there is great variability in the response, ranging from 23 to 86% according to the different centers [67, 68]. Early PTLD tends to be more susceptible to modification of the immunosuppression, but one drawback associated with the recovery of immunocompetence is represented by rejection of the graft. This is of particular concern for recipients of heart or liver grafts who invariably lack alternative therapeutic options unlike kidney recipients who usually have access to dialysis in the event of graft rejection. For localized forms of PTLD, surgical resection is generally successful with complete resolution of the disease. In contrast, alternative therapeutic strategies are required to effectively treat PTLD not responsive to the withdrawal of immunosuppression and/or PTLD presenting as a more aggressive and diffuse disease. Antiviral agents may offer some advantages as preemptive therapy, by reducing the incidence of symptomatic post-transplant seroconversion [69]. Similarly, biological immune-response modifiers like interferon can be used to prevent the infection of new lymphocytes by inhibiting viral replication [19, 70].

Recently, more centers are reporting regression of PTLD in SOT recipients treated with the humanized anti-CD20 monoclonal antibody [71–73]. Complete responses are generally observed for early PTLD, however the data is limited regarding the long-term maintenance of such remissions. Late PTLD seems less responsive to Rituximab. This may be dependent on the heterogeneity of CD20 expression on these tumor cells. In addition, the possibility of selecting CD20-negative PTLD using this treatment needs further evaluation [74]. Finally, more information is required on the long-term effects of multiple anti-CD20 administrations in children, especially on the B-cell compartment recovery.

Chemotherapy represents another treatment option, especially for aggressive disease. Though several groups have reported complete remission after a variety of chemotherapy regimens, severe toxic effects and high mortality rate have also been described [75]. Low-dose chemotherapy regimens appear to provide control of the disease and are well tolerated in terms of treatment-related toxicity and infections [76]. However, as yet there is no uniform approach for the treatment of EBV-driven PTLD in SOT recipients. Adoptive immunotherapy may have therapeutic potential in this setting as previously demonstrated for PTLD developing after HSCT.

Adoptive Immunotherapy Approach in SOT Recipients

The majority of the localized forms of PTLD and the 'early' lesion that presents as polymorphic proliferation can be considered as 'non-cancerous' stages, since regression may be obtained after reduction or withdrawal of the immunosuppression resulting in restoration of the T-cell function. Late-onset PTLD is generally monomorphic and associated with point mutations or rearrangement of proto-oncogenes or tumor suppressor genes [77]. This suggests that the broad array of PTLD observed after SOT reflects a multistep process. Whether delays in detection and treatment of 'early' PTLD may permit progression to a more aggressive malignant disease is not known. Some indications that 'early' PTLD can progress to these malignant lymphomas come from the identification of EBV infection in draining lymph nodes or identification of partial sequences of the EBV genome in some of these lymphomas [78, 79].

The use of EBV-specific CTL to restore EBV-specific immunity early in the disease process may provide the ideal therapeutic strategy to prevent PTLD progressing to a more aggressive, treatment-resistant form. However, the generation of EBV-specific CTL for SOT recipients presents some major differences compared to HSCT recipients. First, SOT recipients and donor are not HLA-matched and PTLD occurring after SOT are of recipient origin, so that the use of donor-derived CTL is not appropriate [80]. Secondly, the grafted organ is generally from a cadaver, which obviously precludes the ex vivo generation of donor-derived CTL. Infusion of CD3+ leukocytes from an HLA-matched sibling has been previously reported for the treatment of PTLD [63]. However, the

availability of such donors may be limited and graft toxicity or rejection may occur. Graft rejection has also been observed after the infusion of autologous natural killer-like T cells, obtained by expansion in vitro in the presence of interleukin-2 [81]. To reduce alloreactivity, the generation of EBV-specific CTL from allogeneic EBV-seropositive healthy donors based on the 'best HLA match' has been proposed [82]. However, the infused CTL seem to be rapidly eliminated by the recipient's T cells.

Autologus EBV-specific T-cell lines therefore represent the best option for the adoptive transfer. Autologous CTL from SOT recipients generated before the transplant procedure are able to restore EBV-specific competence [83]. However, this approach is not feasible, since the number of transplant procedures is increasing and it would be too expensive and time-consuming to generate these T-cell lines for all transplant candidates. Therefore, the optimal approach is to generate CTL only for patients with active disease. This includes patients with PTLD or patients acquiring EBV seroconversion post-transplant. Initially there were concerns regarding patient immunosuppression and the feasibility of generating CTL from these patients. While HSCT donors are healthy subjects, SOT recipients are continuously on immunosuppressive treatment. Khanna et al. [84] first showed that it was possible to generate EBV-specific CTL from SOT recipients who were receiving immunosuppression when the EBV seroconversion occurred. We have confirmed this finding by successfully generating EBV-specific CTL from 2 patients who seroconverted after the transplant procedure. In addition, we have been successful in generating EBV-specific CTL from other 6 SOT patients receiving immunosuppression treatment, even those presenting with active PTLD [85]. By using multiple stimulations with autologous LCL in the presence of interleukin-2 from day +15 of culture, all our patients' CTL expanded easily with normal kinetics, phenotype and cytotoxic activity.

Though feasible, the time required for the establishment of these T-cell lines remains the major limitation of this approach and other treatments are required to allow sufficient time for CTL establishment. Alternatively, preventive CTL generation should be reserved for high-risk patients. To select patients highly immunosuppressed, a test that correlates the patient's immunodeficiency status with the incipient PTLD development is necessary. So far the evaluation of EBV-DNA level as evidence of lack of EBV-specific immune control has been used [14, 86–88]. However, the measurement of EBV-DNA levels does not seem to be specific, since an increase of the EBV-viral load is not always followed by PTLD. In our institution, we are evaluating alternative methods for measuring cellular immune responses to EBV. The availability of a test for the early detection of EBV-PTLD which is rapid, sensitive and specific will give us not only enough time for the establishment of CTL lines but also the opportunity for early intervention.

Another limitation of the adoptive immunotherapy approach is the failure to generate EBV-specific CTL from EBV-seronegative SOT recipients using multiple stimulations with autologous LCL in presence of interleukin-2. In fact, although LCL are potent antigen-presenting cells, they seem incapable of inducing a primary immune response in vitro. In our institution, new strategies for the generation of EBV-specific CTL from EBV-seronegative SOT recipients are currently under evaluation. The possibility to treat symptoms at seroconversion and PTLD in this group of patients would drastically reduce the incidence of this disease.

Conclusions and Future Directions

The use of adoptive transfer represents a physiologic treatment for PTLD in HSCT and SOT recipients, since it allows restoration of the immunosurveillance that is compromised in these individuals. A summary of the studies where CTL therapies have been used for the treatment or prophylaxis of PTLD are listed in table 2. In the SOT setting, treatment of early lesions would prevent the EBV-transformed cells from progressing into aggressive lymphoma. However, further information is required to assess efficacy and persistence of the infused CTL in these patients. Preliminary studies suggest that CTL administered to SOT recipients continuing immunosuppressive therapy are effective in reducing EBV-DNA viral load but do no persist for longer than 3 months after the infusion [83, 84].

In the HSCT setting, the use of EBV-CTL is effective for the treatment and prophylaxis of PTLD. However, the development of tumor escape mutants despite the used of polyclonal CTL lines is a concern. Other approaches such as depleting the stem cell graft of B as well as T cells have been investigated by our group and may be a feasible option especially when donor cells are not available (e.g. post-matched unrelated cord blood transplant).

In summary, CTL therapy may best be used as prophylaxis or for minimal residual disease, as the presence of fewer tumor cells will reduce the risk of tumor escape mutants. However with the time investment required to generate such CTL lines, future directions should be

Table 2. Published studies using adoptive immunotherapy for treatment or prophylaxis of PTLD

Study	Patients (patient age)	Immunosuppression	PTLD	CTL lines	Results
Post-HSCT					
Rooney [35]	56 (9 months to 20 years)	T-depleted HSCT (MMRD or MUD)	Prophylaxis	Allogeneic	No PTLD cf. 11.5% control No toxicity
Heslop [35, 55]	3 (12–17 years)	T-depleted HSCT	Lymphoblastic lymphoma	Allogeneic	2 CR, 1 NR (died) tumor mutation resistant to CTL
Gustafsson [53]	9 (1–39 years)	T-depleted HSCT or ATG/OKT3	↑EBV-DNA	Allogeneic	8 pts ↓EBV-DNA 1 pt died from PTLD (CTL not EBV-specific)
Post solid organ transplant					
Emanuel [63]	1 (11 years)	CSA+AZA+Pred	CNS B-lymphoma	HLA-matched lymphocytes	CR but signs of rejection
Nalesnik [81]	7 (41–61 years)	Not available	EBV +ve (4 pts) EBV –ve (3 pts)	Autologous LAK cell line	3 CR, 3 NR, 1 died of sepsis
Haque [83]	3 (29–61)	FK506 or CSA	Prophylaxis	Autologous	↓EBV-DNA ↑CTLp
Khanna [84]	1 (39)	CSA + AZA + Pred	B-lymphoma	Autologous	↑CTLp and initial CR then died from PTLD

MMRD = Mismatched related donor; MUD = matched unrelated donor; CR = complete remission; NR = no response; ATG = antithymocyte globulin; OKT3 = anti-CD3; CSA = cyclosporin; AZA = azathioprine; Pred = prednisone; LAK = lymphokine-activated killer cell; FK506 = tacrolimus; CTLp = CTL precursor frequency.

focused on identifying high-risk patients in the HSCT and SOT populations so that prophylaxis is timely and effective. Known risk factors such as previous lymphoma, immunodeficiency syndromes, type of immunosuppression as well as the use of quantitative real-time PCR to regularly monitor EBV DNA levels are helpful tools to preempt PTLD development and expedite the appropriate therapy. In addition, the ability to generate autologous EBV-CTL lines in seronegative SOT recipients will be invaluable for the treatment of this large at-risk population.

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Adoptive T-Cell Therapy for Epstein-Barr Virus-Positive Hodgkin's Disease

M. Helen Huls Cliona M. Rooney Helen E. Heslop

Center for Cell and Gene Therapy, Baylor College of Medicine, Houston Tex., USA

Key Words
Epstein-Barr virus · Adoptive immunotherapy · Cytotoxic T lymphocyte · Hodgkin's disease

Abstract
Immunotherapy approaches with antigen-specific cytotoxic T lymphocytes (CTLs) have proved safe and effective prophylaxis and treatment of Epstein-Barr virus (EBV)-associated lymphomas arising after bone marrow transplantation. EBV is also associated with other malignancies including about 40% of cases of Hodgkin's disease making this tumor another potential target for EBV-targeted immunotherapy. While studies with autologous EBV-specific CTLs have shown antiviral activity and immune effects, the clinical responses have been less impressive than those observed in post-transplant lymphomas. There are several possible reasons why the malignant cells in EBV-positive Hodgkin's disease may be less susceptible to immunotherapy approaches, including the fact that they express a more restricted array of EBV-encoded antigens and possess many immune evasion strategies. A number of approaches to overcome these tumor evasion strategies including targeting CTLs to the expressed antigens and genetic modification of CTLs are being evaluated.

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Rationale for Immunotherapy Approaches

Immunotherapy approaches using Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes (CTL) to prevent or treat cancer have been successful in severely immunosuppressed patients, who are at high risk for virus-associated tumors such as EBV-associated lymphoproliferative disease (EBV-LPD) [1–4]. The malignant cells in EBV-LPD are highly immunogenic and express all 9 latent cycle EBV-encoded antigens [5]. EBV-associated cancers also include other lymphoid disorders like Burkitt's lymphoma and Hodgkin's lymphoma and the epithelial cell malignancy nasopharyngeal carcinoma [6]. In Burkitt's lymphoma a much more restricted pattern of gene expression is observed, with expression of the EBNA-1 protein and EBER RNAs (latency I). An intermediate pattern (latency type II) is seen in nasopharyngeal carcinoma and Hodgkin's lymphoma with expression of EBNA-1, BARFO, LMP1 and LMP2 as well as the EBER RNAs. Nevertheless these antigens provide potential targets for immunotherapeutic approaches with CTLs.

About 40% of cases of Hodgkin's disease in North America and Europe are associated with EBV [7]. In South America, Kenya and parts of Asia, the association is 90–100%. Although 80% or more of patients with Hodgkin's disease are cured with available treatments, 50% of the minority who relapse, fail to respond to sal-

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Helen E. Heslop
Center for Cell and Gene Therapy
1102 Bates Street, Suite 1140
Houston, TX 77030 (USA)
Tel. +1 832 824 4662, Fax +1 832 825 4668, E-Mail hheslop@bcm.tmc.edu

vage chemotherapy or relapse a second time. Further, the unacceptably high level of therapy-related secondary malignancies (18% at 5 years) and other serious medical complications in those who are 'cured' also underscore the need to improve current therapeutic options [8]. A number of factors could diminish the effectiveness of EBV-specific CTLs in Hodgkin's disease. First of all, the malignant cells express a restricted set of viral genes, namely, EBNA-1, RK-BARFO, LMP1 and LMP2 [9]. In the majority of cases, memory CTL responses are preferentially directed against the highly immunogenic EBNA-3A, -3B and -3C antigens, depending on the patient's HLA type. By comparison, EBNA-1, BARFO, LMP1, and LMP2 are poorly immunogenic. EBNA-1 contains a repeating glycine-alanine amino acid sequence that inhibits ubiquitination and subsequent processing and presentation of EBNA-1 peptides by class-I human leukocyte antigens (HLAs) [10]. EBV+ Hodgkin's tumors express co-stimulatory molecules such as HLA DR, CD40, CD80 and 86 and should therefore be both excellent antigen-presenting cells (APCs) and good target cells for CTLs. However, they also secrete the TH2 cytokines interleukin (IL)-10 and tumor growth factor-β (TGF-γ) as well as TARC, a chemoattractant for CD4+ T cells of the TH2 biotype. This overwhelmingly TH2 bias likely helps the antibody response and inhibits the CTL response. IL-10 may also act as an autocrine growth factor for Reed-Sternberg (RS) cells. In addition, patients with Hodgkin's disease also have T-cell abnormalities, such as low expression of the ζ chain of the T-cell receptor (TCR) [11], which further reduces the effectiveness of the host immune response to the tumor [12, 13].

Preclinical Studies

To assess the feasibility of using EBV-specific CTLs as therapy for Hodgkin's disease, we generated EBV-specific CTLs from the peripheral blood of patients with this malignancy, with the notion of expanding the specific T cells in vitro in the absence of in vivo immunosuppressive effects. We then compared them phenotypically and functionally with CTLs generated from normal donors [14]. In the presence of IL-2 and B-LCL, cell counts of cultures from healthy donors (n = 15) typically increased by 10-fold every 2 weeks, so that after 16 weeks in culture CTLs from normal donors had expanded approximately 1,500-fold. During the same 16-week period, CTL cultures from patients in remission expanded by only approximately 150-fold, while those from patients with relapsed disease increased by just 80-fold. In more than 75% of patients, however, it was still possible to generate at least 10^8 CTLs, a number suggested by previous studies of EBV-LPD to be well in excess of that required to establish EBV immunity [1, 2]. Phenotypically, the patient lines were essentially identical to the lines from normal donors, except that the level of the TCR-ζ chain was abnormally low [15]. Nonetheless, the lines produced had strong activity against HLA-matched EBV-positive targets. Of particular note, clones recognizing Hodgkin's-associated viral antigens could be detected by LMP-2 tetramers.

More recently by modifying our standard CTL generation procedures to include routine 'superexpansion' with irradiated mononuclear cells (MNCs) and OKT3 and using EHAA medium, we have been able to shorten the CTL generation time. Comparisons were made between RPMI 1640 containing 10% fetal calf serum (FCS; RPMI/FCS) and 45% RPMI 1640 with 45% EHAA and 10% FCS (RPMI/EHAA/FCS). The addition of EHAA consistently and quite dramatically improved CTL expansion. A potential drawback with the use of EHAA medium was that in its presence there was an increased percentage of CD4+ T cells in the CTL line [Huls, unpubl. data].

Preliminary Results of Our Current Trial

We are therefore evaluating autologous polyclonal EBV-specific CTL infusion in 2 federally approved phase-I/II clinical trials for patients with EBV-positive Hodgkin's lymphoma with multiple relapses (group A) or with minimal residual disease after autologous stem cell transplant (group B) [16]. Of 94 referred patients 39 (42%) had EBV-positive tumors as measured by expression of viral small RNAs (EBERs) or LMP1. EBV-transformed lymphoblastoid cell lines (LCLs) were generated in 30 patients and in most cases CTLs were successfully generated. Four patients with advanced disease died before the generation of LCLs and CTLs was completed. Eight patients with relapsed disease (group A) have been treated on study and have either received 4×10^7 (n = 6), or 2×10^7 CTLs/m^2 (n = 1) or 1.2×10^8 CTLs/m^2 (n = 1). Seven patients in group A received CTLs marked with a retroviral vector encoding the neomycin resistance gene (Neo), so their fate and persistence in vivo could be tracked [16]. Three patients have been treated in group B and received two injections of unmarked CTLs at a dose of 2×10^7/m^2. No immediate toxicity was observed after CTL infusion in either group A or B. In group A, 6 patients with aggressive disease at the time of CTL infusion survived for 8–

18 months, 1 patient had tumor erosion through the left upper lobe bronchus and died 2 months after CTL infusion, and 1 patient is alive 25 months after infusion. The 3 patients in group B are alive and well 9–16 months after CTL infusion. Studies using an LMP-2 tetramers in HLA A2-positive recipients showed an increase in frequency of positive cells after CTL infusion. The frequency of interferon (IFN)-γ-producing CD8 T cells in response to LCL also increased after CTL infusion. Gene-marked CTLs were found in the peripheral blood up to 9 months following infusion. Neo-marked cells and LMP-2 tetramer-specific T cells were found localized to a malignant pleural effusion in 1 patient 3 weeks after CTL infusion [15]. This study demonstrates that: (1) adoptive immunotherapy with autologous EBV-specific CTLs is well tolerated in patients with Hodgkin's disease; (2) CTLs can localize to the Hodgkin's tumor; (3) gene-marked CTLs persist for up to 12 months after infusion, and (4) LMP2-specific CTLs are increased in the peripheral blood after CTL infusion and strategies to increase their frequency may result in a more specific cytotoxic response against EBV+ Hodgkin's tumors.

Possibilities for Improving Immunotherapy Approaches

Targeting CTLs towards Subdominant Antigens Expressed by Tumor

Lack of CTL efficacy may simply be quantitative in that the current method of EBV-specific CTL generation activates too few clones reactive with the viral proteins expressed on Hodgkin's tumor cells. Instead LCLs activate CTL lines that are dominated by clones reactive to the immunodominant EBNA-3A, 3B and 3C viral proteins that are not expressed in Hodgkin's lymphoma. Of the 4 EBV-associated antigens expressed in H-RS cells, only LMP1 and LMP2 are potential antigens for the generation of antigen-specific CTLs. EBNA-1 is not processed for HLA class-I presentation, and attempts to identify BARFO-specific CTLs have produced lymphocytes that recognized target cells expressing BARFO at a high level, but failed to recognize the antigen expressed at low levels in lymphoblastoid cell lines. LMP1-specific CTL clones are rare in EBV-positive donors [17] and very few LMP1 epitopes have been identified [18]. Moreover LMP1 displays heterogeneity between virus strains [19] and CTL raised against B cell (B95–8 prototype)-derived LMP1 may not recognize the LMP1 tumor variants [20]. Thus we and others have focused on the generation of LMP2-specific CTLs. In preclinical studies several different methodologies have been used to generate LMP2-specific CTLs [21–24]. Using either peptide-pulsed dendritic cells [23], dendritic cells transduced with adenovirus [21, 24] or LMP2 RNA [22], it is possible to preferentially expand LMP2-specific CTLs when compared to the standard protocol which uses LCL as the APC. Because of these encouraging in vitro results we are currently modifying our clinical protocols to administer LMP2-specific CTLs for patients with relapsed EBV-positive Hodgkin's lymphoma. An alternative method of isolating LMP2-specific CTLs is by cloning from polyclonal lines but this is highly labor intensive due to the low frequency of LMP-2-specific CTLs in such lines [17], and is likely to produce CTLs with a limited target epitope repertoire. Finally a recent report describes transfer of CTL activity from a LMP2 peptide-specific CTL clone to a stimulated peripheral blood mononuclear cell (PBMC) population by transducing the PBMCs with a retroviral vector encoding the appropriate TCR [25].

Genetically Modifying CTLs to Overcome Tumor Evasion Strategies

Although the malignant RS cells in Hodgkin's disease appear to be good APCs, they have additional immune evasion strategies that act later in the process of CTL generation [26]. They secrete IL-10, an anti-inflammatory cytokine that deactivates professional APCs and thus prevent them both from cross-priming and recruiting tumor-specific CTLs. They secrete TGF-β which inhibits T-cell activation and expansion by inhibiting early signaling events essential to the induction of IFN-γ, IL-12 and TNF [27, 28] They also secrete the chemokine, TARC, which selectively recruits Th2 cells, which in turn release IL-4 and inhibit the Th1 CTL response, likely explaining why patients with Hodgkin's disease produce tumor-specific antibodies [29–31]. The idea that the RS cells generate a Th2 environment is supported by the observation that the T-cell infiltrate consists exclusively of CD4+ T cells with a Th2 profile [32]. Finally RS cells express the ligand for the death receptor Fas. Since Fas is expressed on activated CTLs, apoptosis may be induced in the CTLs on interaction with the RS cell. This would not only prevent the CTLs from killing other tumor cells, but also preclude their expansion in tumor tissues. The fact that Hodgkin's cells need multiple mechanisms to evade immune responses suggests that one or more single mechanisms could be overcome. We are currently evaluating whether introduction of a dominant negative TGG-β receptor can render CTL resistant to the inhibitory effects of TGF-β.

Conclusions

Initial immunotherapy studies with EBV-specific CTLs in patients with EBV-positive Hodgkin's disease have shown evidence of immune activity and partial clinical responses but no complete clinical responses. Follow-up studies will focus on targeting CTL to the subdominant EBV-encoded antigens expressed by this tumor, while preclinical studies are evaluating whether tumor evasion mechanisms can be circumvented by genetically modifying CTLs.

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Chimeric T-Cell Receptors for the Targeting of Cancer Cells

Claudia Rössig[a] Malcolm K. Brenner[b]

[a]University Children's Hospital Münster, Department of Pediatric Hematology and Oncology, Münster, Germany;
[b]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Tex., USA

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Adoptive immunotherapy · Cytotoxic T cells · T cell receptor · Costimulation · Gene therapy

Abstract
Genetic engineering of human T lymphocytes to express tumor antigen-specific chimeric immune receptors is an attractive means for providing large numbers of effector cells for adoptive immunotherapy. Major mechanisms of tumor escape from immune recognition are efficiently bypassed. Although adoptive transfer of chimeric receptor-expressing peripheral blood-derived T lymphocytes has produced some anti-tumor activity in mice, the first clinical studies have revealed a disappointing lack of correlation between in vitro cytotoxicity and therapeutic efficacy. The most pertinent issue is that chimeric T cells fail to expand and rapidly lose their function in vivo. Potential strategies to enhance the therapeutic value of chimeric receptor-modified cells by preventing their functional inactivation in vivo are currently being investigated.

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Specific cytotoxic T cells (CTL) are important mediators of the physiological immune defense against eukaryotic cells, such as allogeneic or virus-infected cells. There has been increasing interest in the use of CTL for the treatment of cancer. Tumor cells which present tumor-specific protein antigens on molecules of the major histocompatibility complex (MHC) can indeed be lysed by ex vivo generated CTL with native specificity for the respective peptide. This strategy has been successfully used for the treatment and prophylaxis of Epstein-Barr-virus-associated lymphoproliferative disease and lymphoma [1–3]. However, attempts at generating CTL against non-viral antigens by selection and expansion of T cells in the presence of autologous tumor cells have generally failed. A likely explanation is the lack of tumor specificity of most tumor antigens. In contrast to viral antigens, most tumor-associated proteins are co-expressed on normal cells or at certain developmental stages. According to the rules of immune tolerance towards self, these antigens cannot be expected to induce efficient immune activation. Furthermore, efficient anti-tumor T-cell activity is complicated by the nature of antigen recognition by T cells, requiring the processing of cellular proteins into peptide fragments, their association with molecules of the MHC and their transport to the cell surface. Many tumor cells have

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www.karger.com/aha

Claudia Rössig, MD
University Children's Hospital Münster
Department of Pediatric Hematology and Oncology
Albert-Schweitzer-Strasse 33, DE–48149 Münster (Germany)
Tel. +49 251 8352819, Fax +49 251 8352804, E-Mail rossig@uni-muenster.de

acquired defects of these mechanisms for efficient antigen presentation which allows them to escape immune attack.

In an attempt to extend the recognition specificity of T lymphocytes beyond their classical MHC-presented peptide targets, a gene-therapeutic strategy has been developed that allows us to redirect T cells to antibody-defined targets. This novel concept is based on structural similarities between the recognition domains of an immunoglobulin molecule and the T-cell receptor. Antibodies recognize their targets through hypervariable regions located within the variable (V) domains of their heavy and light chain. T-cell recognition is mediated by regions within the α and β chain of the T-cell receptor that share structural homology with the antibody V domains. Receptor engagement by specific peptide presented on MHC results in tyrosine phosphorylation of immune-receptor activation motifs present in the cytoplasmic domain of the ζ chain component of the T-cell receptor, initiating T-cell signaling to the nucleus and exertion of tumor cell-directed effector functions.

In a first attempt to endow T cells with antibody-type specificity, hybrid receptors were created in which the α and β chain V domains were replaced with the corresponding portions of the heavy and the light chain V domains [4–7]. The modified T-cell receptor chains were functionally expressed in T cells and recognized antigen in a non-MHC-restricted manner. With these experiments, it became evident that antibody-derived V_H regions can replace a Vα or Vβ region to form functional receptors.

Based on the observation that proteins belonging to the ζ receptor family are capable of mediating complete signals that suffice to induce immune effector functions [8–10], receptors were then constructed that directly link the V domains of a monoclonal antibody to a cytoplasmic immune receptor domain (fig. 1). Several receptor designs employing the signal-transducing intracellular components of either the Fc receptor or TCR have been tested. To allow for functional recognition of the target antigen, the receptors were spaced from the plasma membrane, usually by insertion of an immunoglobulin hinge-like domain. Gene transfer into human or murine T lymphocytes was performed by transfection or using retroviral vectors. T cells engineered to express the recombinant chimeric receptor genes were shown to be capable of producing specific lysis and cytokine secretion upon exposure to tumor cells expressing the respective target antigen on their surface [11–14]. Adoptive transfer of these cells in tumor models in mice have shown significant anti-tumor activity of the genetically modified effector T cells in vivo [13, 15].

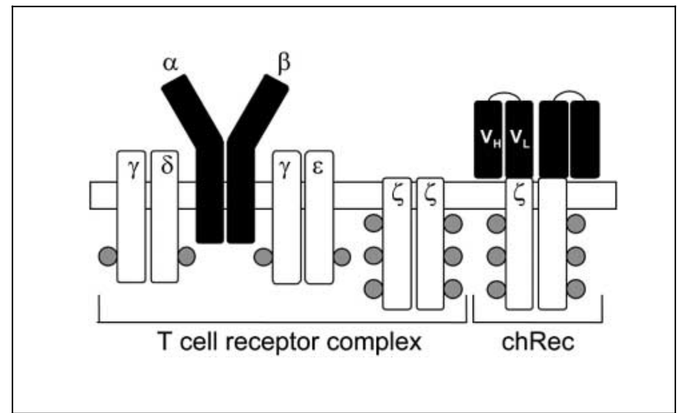

Fig. 1. Structure of the T-cell receptor and a chimeric receptor.

The use of chimeric receptor-transduced T cells for the treatment of cancer has numerous theoretical advantages over classical T-cell-based immunotherapies. As opposed to the lengthy process of in vitro selection, characterization and expansion of T cells with native specificity for tumor-associated antigens, genetic modification of polyclonal T cells allows to generate high numbers of tumor-specific T cells within weeks. Since antigen recognition by chimeric receptors is independent of MHC restriction and antigen processing, major mechanisms of tumor escape from immune recognition are efficiently bypassed. Importantly, this strategy is applicable to every malignancy that is associated with a tumor antigen for which a monoclonal antibody exists, including non-protein antigens, and thus extends the spectrum of malignancies eligible for T-cell-based immune therapy by a multitude of solid tumors and hematopoietic malignancies. Tumor antigens used as targets for chimeric receptors include Neu/HER2, folate-binding protein (FBP), CEA, TAG-72, renal tumor-associated antigen, epithelial glycoprotein-2, CD30, CD33, G_{D2} [16, 17], and angiogenic endothelial cell receptor (KDR) [12, 13, 18–23]. Moreover, receptors have been generated against HIV [24–27] and against targets involved in autoimmune diseases [28, 29].

In recent years, attempts have been made at translating preclinical experiences with chimeric receptor-transduced T cells into clinical trials. One of the first chimeric receptor cell therapies tested in humans was an adoptive immunotherapy protocol targeting HIV [30, 31]. A chimeric receptor bearing the extracellular domain derived from the HIV receptor CD4 had been shown to be func-

tionally active in vitro by redirecting T cells to efficiently lyse CD4+ T cells infected with HIV [26].

Adoptive transfer of ex vivo expanded CD4ζ-modified syngeneic CD8+ T cells in HIV-infected twin pairs was well tolerated. Clinical or virological effects were not seen in any of the treated patients. The lack of anti-retroviral activity was associated with a rapid decline in gene-marked cells in the blood following the cell transfusions [31]. According to viral models, persistent control of tumor growth by CTL likely requires sustained in vivo proliferation of the transferred cells and their repeated expansion in response to encounters with tumor cells. The key issues determining in vivo survival and persistent anti-tumor functionality of gene-modified CTL are their immunogenicity, their requirement for specific T-cell help and co-stimulation, and the quality of chimeric receptor signaling.

Rapid clearance of modified T cells may partly be overcome by reducing the immunogenicity of the recombinant receptors. Currently available hybridoma antibodies can be humanized by replacing murine framework regions [32, 33], and fully human recombinant single-chain antibodies can be generated by phage display technology [34]. Co-expression of potentially immunogenic markers genes can be avoided by optimizing the efficiency of retroviral gene transfer systems for T-cell transduction, eliminating the need for selection of transduced cells [35].

The in vivo requirements for disease control by T cells have been studied in murine models of viral infection. Specific T-helper cells have been shown to be of critical importance for the maintenance of CTL function. In situations in which CD4 help is deficient, CTL responses may be silenced [36]. Thus, co-transfusion of antigen-specific CD4+ T cells along with the transduced CTL may be necessary for long-term maintenance of adoptively transferred T cells. Prolonged survival of genetically engineered HIV-specific CTL for at least 1 year was indeed achieved by co-transfusion of CD8+ and CD4+ chimeric receptor-modified T cells [30]. Disappointingly, however, the cells again did not appear to have an anti-retroviral effect in vivo, even though the cells had high levels of in vitro activity against ovarian cancer. In an additional clinical trial at the NCI, patients with ovarian carcinoma are treated with FBP-specific chimeric receptor T cells. In the first 8 patients treated with up to 5×10^{10} cells, no clinical responses were seen [37]. Thus, providing T-cell help does not seem to suffice for persistent effector function of chimeric receptor CTL.

A key question is whether signaling via the chimeric receptor provides an activation signal of sufficient strength and quality for inducing adaptive immune responses in the transduced T cells. Ideally, analogous to T-cell priming via peptide/MHC, engagement of chimeric receptors by tumor cells would induce a complete immediate effector response as well as specific T-cell memory, establishing efficient and life-long anti-tumor immunity. However, studies performed in transgenic mice suggest that the function of chimeric receptor proteins depends upon the activation status of the T cell. Chimeric receptor-mediated signaling was not sufficient to trigger activation of resting primary T cells unless they had been prestimulated through their native receptor [38, 39]. In accordance, in vitro stimulation of T cells via chimeric receptors does not induce a proliferative response [40–42]. These observations suggest that chimeric receptor signaling is limited. Whereas effector functions are efficiently induced, signals that are required for induction of clonal proliferation of activated T cells are incomplete. Although chimeric receptors can serve as surrogates for the T-cell receptor, they differ from the native T-cell receptor in important aspects. The cellular microenvironment of the immunological synapse, which is responsible for efficient recruitment of engaged receptors into kinase-rich microdomains, contributes significantly to sustained signaling, as necessary for complete T-cell activation. Detailed knowledge about the molecular mechanisms and deficits of chimeric receptor-mediated signal transduction are not available to date. It has been suggested that the restricted subset of T-cell receptor signaling domains present within chimeric receptors might be responsible for the observed functional deficits. However, the number of immune receptor activation motifs (ITAMs) present within the intracellular portion of the receptor does not seem to correlate with the receptor function: the T-cell receptor ζ chain, containing three ITAMs, and the Fc receptor γ chain with one single ITAM only were both used as chimeric receptor signaling domains with comparable effectiveness [8, 11, 14, 27]. Efforts have been made to develop clinically effective chimeric receptors with improved signal transduction characteristics. In an attempt to bypass co-receptor engagement, receptors were constructed in which the src family kinase lck was directly linked to the ζ chain in a combined receptor. Engagement of the combined receptor promoted formation of a qualitatively superior signal-transducing complex, reflected by enhancement of early events in TCR signal transduction and in a greater quantity of IL-2 release. These new receptors have not been validated clinically yet.

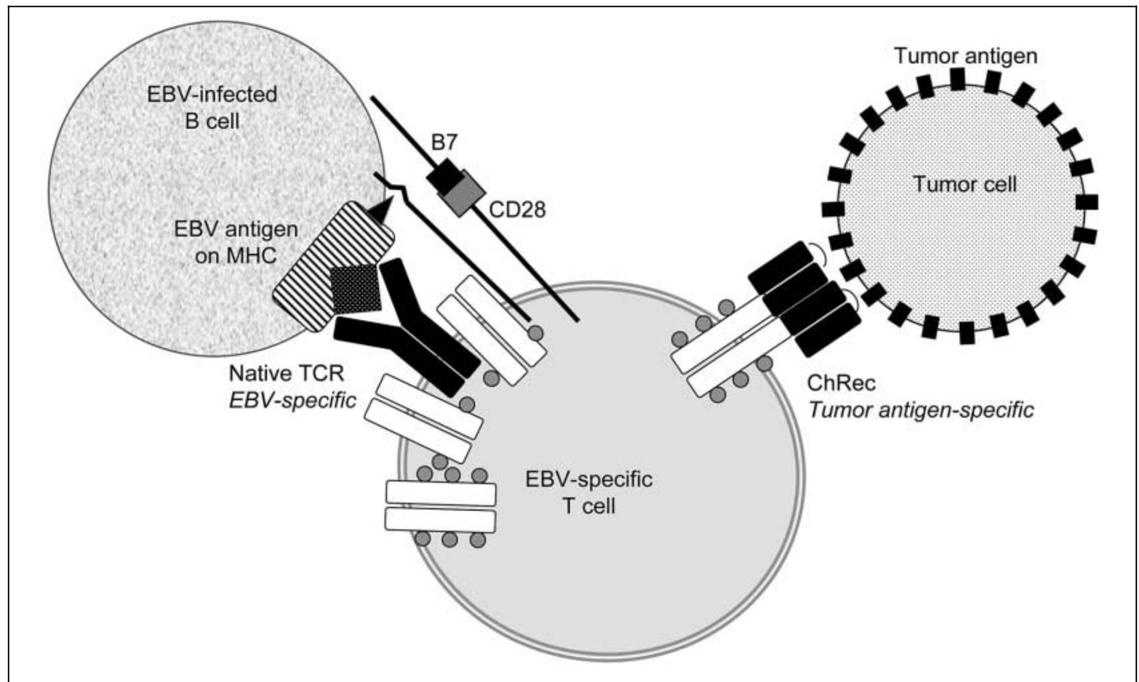

Fig. 2. Model: Use of EBV-infected B lymphocytes and chimeric T-cell receptors to target cancer cells. CD8 T cells bearing a tumor-specific chimeric TCR are activated by EBV antigen binding to a native TCR and are co-stimulated through the interaction of B7/CD28. They may receive additional cognate help from EBV-specific CD4+ T cells. Tumor cell lysis is mediated by the chimeric receptor.

The functionally inactivated phenotype of chimeric receptor CTL can also be explained by the absence of appropriate co-stimulation. According to the dual signal model of T-cell activation, in addition to the signal transmitted via the T-cell receptor, a co-stimulatory signal is required for efficient priming of naive T cells, resulting in cellular proliferation, secretion of cytokines, and prevention of activation-induced anergy [43]. The best known co-stimulatory pathway is the interaction between B7 and CD28. Physiologically, CD28 co-stimulatory signals are provided by professional antigen-presenting cells. As the majority of tumor cells do not express B7, co-stimulatory signals are not expected to be transduced when the chimeric receptor is engaged. In vitro experiments have shown that chimeric receptor-mediated induction of cytokine secretion and T-cell proliferation is substantially enhanced by co-stimulator signaling [41, 44, 45]. CD28 thus seems to contribute to efficient T-cell activation via chimeric receptors. The in vivo functionality of chimeric T-cell receptors in situations where co-stimulation is limited remains to be determined. Modified chimeric receptor designs aim at co-transmitting co-stimulatory signals along with the chimeric receptor signal.

Effective CD28 signaling can be mediated in primary T cells by chimeric receptors fusing an extracellular anti-tumor antibody fragment to the signal transduction domain of CD28 [23]. Double transfectants simultaneously expressing scFv-CD28 and scFv-CD3ζ chimeras result in effective co-stimulation when binding to the antigen recognized by both scFv [46]. In an attempt to co-deliver co-stimulation in a single receptor, chimeras were constructed that combine the signaling domains of the T-cell receptor ζ chain and the CD28 receptor, linked to an extracellular anti-tumor antibody domain [41, 45, 47, 48]. In transduced T cells, both signals were indeed transduced through the same receptor, resulting in efficient co-stimulation.

An alternative approach to overcoming the limitations of current chimeric receptor immunotherapies is the genetic engineering of an effector T cell with native specificity for a strong viral antigen (fig. 2). Epstein-Barr virus (EBV) infection of B lymphocytes is near universal in humans and stimulates high levels of EBV-specific helper and CTL, which persist indefinitely. Clinical studies have shown that EBV-specific T cells generated in vitro will expand, persist and function for more than 6 years in

vivo. Following genetic modification with anti-tumor chimeric receptor genes, EBV-specific T cells can be expanded and maintained long term in the presence of EBV-infected B cells. They recognize EBV-infected targets through their conventional T-cell receptor, and tumor targets through their chimeric receptors, and they efficiently lyse both [49]. Their therapeutic value is still awaiting clinical investigation.

In conclusion, the genetic engineering of T lymphocytes to target antigenic structures on tumor cells via chimeric receptors has high potential for immunotherapy of cancer. Direct application of the experimental design to the clinical setting still requires optimization of individual components of the approach. Long-term functional persistence of transferred T cells appears to be the key issue for the development of successful adoptive immunotherapy with chimeric receptor-engineered cells.

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Leukemia Vaccines

Ludmila Glouchkova Birgit Ackermann Dagmar Dilloo

Clinic for Pediatric Oncology, Hematology and Immunology, University Clinic, Heinrich Heine University, Düsseldorf, Germany

Key Words
Leukemia · Vaccine · Gene transfer · Dendritic cells

Abstract
Evidence that immunological effector mechanisms contribute to the elimination of leukemic blasts in allogeneic bone marrow transplantation supports the concept that the immune system plays a prominent role in the control of leukemic disease. For patients with high-risk acute leukemia, relapse prevention in the setting of minimal residual disease is paramount. This review discusses vaccine strategies aimed to stimulate a leukemia-specific immune response in vivo.

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Leukemia as a Target for Immunotherapy

In leukemia, remission can be achieved with the current-intensified radio-/chemotherapy protocols. For patients suffering relapse, allogeneic stem cell transplantation represents an effective treatment option. Following allogeneic stem cell transplantation, the graft-versus-leukemia effect provides evidence that the immune system contributes to the elimination of residual leukemic blasts [1–7]. Also, identification of leukemia-specific antigens that have been successfully targeted by T-cell responses supports the concept that the immune system plays a prominent role in the control of leukemic disease. Among these leukemia antigens are fusion products of leukemia-specific chromosomal translocations [8–12], antigens that are markedly overexpressed in different types of leukemia [13, 14] but are not truly leukemia-specific and so-called 'shared' antigens that are expressed in different malignancies but not in normal tissue [15–18]. In addition, cytotoxic T-cell clones specific for minor histocompatibility antigens presented by class-I major histocompatibility complex (MHC) molecules have been shown to lyse lymphoid and myeloid leukemic cells [19–22]. Yet, while leukemic cells do express specific antigens that can serve as target structures for anti-leukemic immune responses, in the clinical setting acute leukemias may still escape immune surveillance.

In principle, for effective T-cell stimulation two signals are required [23, 24]. The first signal is mediated via recognition of the antigen-MHC-I complex by the respective T-cell receptor. The second signal is delivered by co-stimulatory molecules such as CD80 or CD86 [23–26]. Co-stimulation may also be provided in a paracrine fashion via cytokines [27]. In the absence of such secondary signals T cells are rendered anergic to the presented antigen [23]. Thus deficient immunogenicity of leukemia cells may in principal be due to defective antigen-processing and/or antigen-presentation or lack of co-stimulatory molecules. As leukemia cells generally express MHC-I molecules to high levels, their reduced T-cell stimulatory capacity is largely attributed to deficient co-stimulation.

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PD Dr. med. Dagmar Dilloo
Klinik für Kinder-Onkologie, -Hämatologie und -Immunologie
Heinrich-Heine-Universität Düsseldorf, Moorenstrasse 5
DE–40225 Düsseldorf (Germany)
Tel. +49 211 811 6103, Fax +49 211 811 6436, E-Mail dilloo@uni-duesseldorf.de

Vaccine Strategies

Vaccine strategies aim to compensate for the reduced immunogenicity of leukemic cells. To this end, vaccine cells are generated in large numbers to express leukemia-specific antigens in the context of adequate co-stimulation. Vaccine cells are then administered via different routes of application with the aim to stimulate a systemic leukemia-specific immune responses. Two principal strategies have been pursued in leukemia vaccine generation. The first strategy aims to improve the antigen-presenting capacity of leukemia cells themselves. This may be achieved either by transfer of genes into leukemia cells that encode the necessary co-stimulatory molecules [28–47] or by induction of leukemia cell maturation via receptor-ligand interactions [48–60]. The second strategy takes advantage of the superior T-cell stimulatory capacity of professional antigen-presenting cells (APCs) such as dendritic cells (DCs) [61, 62]. For delivery of leukemia-specific antigens, DCs generated ex vivo from monocytes [63, 64] or CD34+ hematopoietic progenitor cells [65–67] may be pulsed with leukemia cell lysates or synthetic peptides derived from defined leukemia-specific antigens. APCs are also the major mediators of anti-neoplastic responses stimulated by vaccine cells expressing the granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF has been shown to attract bone marrow-derived DCs to the vaccination site enhancing uptake, processing and presentation of leukemia antigens by professional APCs [68]. Similarly, recruitment of effector cells to the vaccination site promoting the encounter with the relevant antigens and co-stimulatory signals improves vaccine efficacy. Due to their capacity to regulate migration of specific leukocyte subsets [69], chemokines have recently moved into the focus of immunological research and have been successfully employed in a leukemia vaccine model [37].

In leukemia vaccine design, the choice of a particular vaccination strategy depends on conceptual as well as technical considerations. The use of modified leukemic blasts has the advantage that the vaccine cells closely resemble the target cells of the anti-neoplastic response and that the entire repertoire of antigens expressed in the leukemic cells is presented independent of prior identification of leukemia-specific antigens. Technically, sufficient numbers of leukemic cells are readily obtained from bone marrow or peripheral blood. Yet, maintenance of leukemic cells in culture – acute B cell leukemia in particular – and high efficiency gene transfer remain a challenge. In vaccine strategies based on DCs, generation of vaccine cells ex vivo is technically achievable and delivery of leukemia-specific antigens does not necessarily require gene transfer approaches circumventing the potential risks associated with viral transgene delivery.

Improving the Antigen-Presenting Capacity of Leukemic Cells

Genetic Modification of Leukemic Cell

The most efficient methods of transferring genes into somatic cells involve viral vectors. For clinical use, safety-modified derivatives of wild-type virus are generated that carry the transgene but lack the viral genes essential for replication and packaging such that these defective vectors are capable of transgene delivery but not propagation. These vectors can only be generated in producer cell lines providing the necessary viral genes in trans. Prior to any clinical application of such viral vectors, extensive safety testing is required to ensure the absence of replication-competent viral particles, which can emerge by recombination events between the viral vectors and the viral genes inserted into the producer cell line or provided by co-transfected replication-competent helper virus. At present, various viral systems have been approved for clinical trial, namely, retroviruses, adeno-associated viruses, adenoviruses, and herpes simplex viruses. These vector systems differ in titers of viral stocks, tropism, immunogenicity and their capacity to carry large-size inserts, to transduce quiescent cells and mediate transgene integration into the host cell genome [70–73]. High titer vectors allow for bulk transduction of leukemic cells as required for multiple vaccinations and the ability to carry large-size inserts is advantageous when aiming to deliver a combination of immunomodulatory genes. Also, for vaccine applications transient expression of transgenes is usually sufficient. Immunogenicity of viral proteins expressed in target cells transduced ex vivo is acceptable or potentially beneficial adding to the immunostimulatory capacity of the vaccine.

Adenoviral or herpesviral (HSV) vectors exhibit all of these characteristics. Adenoviral vectors have been shown to efficiently transduce chronic myelogenous and activated chronic lymphocytic leukemic cells [74, 75]. Adenoviruses are non-enveloped double-stranded DNA viruses that can be generated to high titers [76]. Adenoviral vectors do not require target cells to be in cycle and transgenes are strongly expressed after adenoviral transduction. Safety modifications consist of deletions of the essential viral genes such as the E1 and/or E3 genes or in

the use of 'degutted' adenoviral vectors lacking all the essential viral genes [73]. Infectivity of adenoviral vectors is determined by target cell expression of the primary Coxsackie-adenovirus receptor as well as expression of α integrins mediating adenoviral internalization [77, 78]. Transduction efficiencies of 50–80% can be achieved in primary chronic myelogenous leukemia (CML) [74]. The efficiency of adenoviral gene transfer into acute myelogenous leukemia (AML) is variable depending on cytokine-induced modulation of adenoviral receptor expression [79, 80] but can be significantly improved by the use of virus polycation complexes [81]. Also retargeting of adenoviruses to cellular receptors, such as heparan sulfates [80], integrins [82, 83] and FC receptors [84], may prove beneficial as shown for monocytes and chronic lymphocytic leukemia (CLL). In CLL, adenoviral gene transfer is significantly enhanced by pre-activation with CD40 Ligand (CD40L) or interleukin (IL)-4 [74, 75] resulting in upregulation of integrin expression. In resting T or B lymphocytes [85] expression of integrins is low accounting in part for the reduced efficiency of adenovirus-mediated gene transfer in non-activated lymphocyte populations.

Here, HSV vectors have proven useful. In acute lymphoblastic leukemia (ALL), gene-transfer rates of 60–80% can be achieved [28] with HSV-1- and HSV-2-derived vectors alike. The Herpesviridae are a family of viruses, which have a large genome of linear, double-stranded DNA (>150 kb) [86–89]. Safety modifications of HSV vectors include deletions of viral glycoprotein H mediating viral infectivity [28, 90] as well as deletions of viral genes essential in replication such as ICP 4, 22, 27 [91]. In acute leukemias, high transduction efficiency achieved with HSV vectors is associated with considerable cellular toxicity. In contrast in CLL [64] that is also highly sensitive to HSV infection, cytopathic effects are less pronounced which has been attributed to high levels of the anti-apoptotic protein bcl-2 in CLL [92]. Yet, while expression of HSV protein results in cellular cytotoxicity, it also enhances vaccine immunogenicity [64, 93].

As no viral proteins are expressed in the target cell following retroviral gene transfer, cellular toxicity and immunogenicity are low. Retroviral transduction results in integration of the gene of interest into the host cell genome facilitating long-term transgene expression yet with the risk of insertional mutagenesis. Application of retroviral vectors for leukemia vaccine generation is further limited by the requirement of target cells to be in cycle as well as by the restriction to a 6–8-kb insert size. Nonetheless, retroviral gene transfer has proven highly effective in CML cells particularly when performed in the presence of fibronectin fragments known to enhance retroviral transduction by co-localization of retroviral particles and hematopoietic target cells [94, 95]. In AML or ALL in contrast, transduction efficiency is highly variable and often low due to the poor proliferative potential of primary human acute leukemia cells in culture [30, 38, 79]. Recently members of two other retroviral subfamilies, the Lentiviridae (HIV1 and 2) and the Spumaviridae (foamy viruses) have attracted considerable attention in the experimental setting [96–98]. The human immunodeficiency virus 1 (HIV-1) vectors have been shown to effectively transduce non-proliferating cells in vitro [99–102]. Primary ALL and AML cells are both transduced by lentiviral vectors [103, 104] and although variation in transduction efficiency at lower multiplicities of infection is considerable, at very high multiplicities of infection (100–3,000) transduction was consistently successful with gene transfer rates between 30 and 80% [36].

Genetically Modified Leukemia Cells as Vaccine Cells

Enhancement of the T-cell response consists of at least three distinct components: (1) attraction of sufficient numbers of lymphocytes to allow for engagement with an antigen-specific T-cell receptor; (2) delivery of a second signal via co-stimulatory molecules, and (3) amplification of the attracted, stimulated effectors cells. Chemokines, cytokines and co-stimulatory molecules may mediate these effects, respectively.

Cytokines

Initial vaccine models for solid tumors employed cytokine-transduced tumor cells as a vaccine. This approach is based on the rationale that paracrine secretion of cytokines in the immediate vicinity of tumor antigens results in the induction of a tumor-specific immune response rather than generalized immune stimulation as observed after systemic cytokine application. Indeed, in a variety of different solid tumor models, vaccine cells transduced with an array of different cytokines with stimulatory activity on effector cells such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and IL-2, 4 and 7 [27, 105–110] have demonstrated convincing efficacy even for eradication of pre-established disease [108, 110]. These treatment models are the most stringent models of tumor vaccination, as the clinical situation of residual disease is closely mimicked. For eradication of pre-established leukemia, in murine vaccine models the single cytokine

approach – with the exceptions of GM-CSF [28, 29, 31, 32] and IL-12 [43, 44, 111] – has been inferior to transgenic expression of co-stimulatory surface molecules such as CD80 [29] and survival is generally improved when combinations of co-stimulatory molecules are employed.

As a single cytokine, transgenic expression of GM-CSF has proven superior to other cytokines in a number of solid tumor vaccine models [112]. GM-CSF lacks primary activity on lymphocytes but induces cross-priming events at high concentrations enhancing uptake, processing and presentation of tumor antigen by professional APCs. High levels of GM-CSF production seem to be critical for the protective effects of GM-CSF-secreting vaccines, yet are difficult to achieve in genetically modified primary human leukemia cells [28, 36]. In some murine leukemia models, vaccine cells secreting high levels of GM-CSF [29, 31, 32] did suppress outgrowth of pre-established leukemia. In other treatment models, anti-leukemic protection mediated by GM-CSF-secreting vaccines was incomplete [28, 30] but, however, could be enhanced by combination with vaccine cells expressing additional immunomodulatory molecules such as lymphotactin, CD80 or IL-12 acting on different levels of the immune response [34, 35, 37].

In murine AML, vaccination with leukemia cells genetically modified to express IL-12 as a single cytokine protects mice against subsequent leukemic challenge and induces rejection of pre-established disease [43, 44, 111]. In a murine ALL model, survival was significantly improved when GM-CSF-secreting vaccine was combined with systemic IL-12 application [33]. In this ALL model, systemic IL-12 administration also enhanced the efficacy of CD80/CD40L-expressing vaccines such that mice with pre-established disease were protected long-term, the protective effects being CD8- and natural killer cell-dependent [33].

Co-Stimulatory Surface Molecules

Transgenic expression of the co-stimulatory surface molecule CD80 also enhances the immunogenicity of myeloid and lymphoid leukemia cells [30, 38, 39, 41, 113, 114]. The potency of CD80 as a co-stimulator is documented in a murine ALL model, with CD80 being the only immunomodulator mediating complete rejection of leukemia in 50% of mice while IL-2 or GM-CSF induced prolonged survival only [30]. In vitro, retroviral transduction of murine AML cells with CD80 enhances expansion of leukemia-reactive cytotoxic T lymphocytes mediating graft-versus-leukemia reactions when infused after syngeneic bone marrow transplantation [39]. Also in a vaccine approach, leukemic cells transgenically expressing CD80 serve to eliminate minimal residual disease; however, efficacy is lost when the vaccine is administered at a later stage of the disease [41, 114]. In keeping with the significance of combination vaccines in leukemia, vaccination efficacy is improved when CD80 is combined with other immunomodulators [32, 34, 35, 115].

Another potent co-stimulatory surface molecule is CD40L which is transiently expressed on activated T cells [116, 117]. Its counter-receptor can be found on B cells, monocytes and DCs [118, 119]. Generally, CD40L–CD40 interactions are critical for interaction between T cells and APCs, B cells in particular [120–122]. Engagement of the CD40 receptor on normal B cells induces differentiation and survival of mature B cells promoting immunoglobulin class switch, antigen-processing, increased MHC expression and upregulation of co-stimulatory surface molecules such as CD80 and CD86. CD40L thus enhances the antigen-presenting capacity of mature B cells [123, 124]. More than 80% of B-lineage CLLs, hairy cell leukemias and ALLs express CD40 [125]. Thus, in the context of leukemia vaccines, CD40L may serve a dual purpose providing both T-cell help and delivery of a maturation signal to B-cell leukemia or DCs serving as a vaccine. In a murine leukemia vaccine model, CD40L-transduced fibroblasts admixed with non-transduced leukemia cells stimulated a protective anti-leukemic immune response in mice with pre-established disease [126], documenting that efficacy does not depend on expression of CD40L on the entire vaccine cell population [126]. As in the murine model, transgenic expression of CD40L in primary human CLL cells via adenoviral or HSV transduction results in upregulation of co-stimulatory molecules [45, 46, 64]. Following infusion of autologous CD40L-transduced CLL cells in a clinical phase-I study, induction of CD80 and CD86 expression on non-infected bystander leukemia cells and an increase in frequency of leukemia-specific T cells was observed. In some patients there was also a decrease in the absolute leukemia cell count associated with a reduction in lymph node size [46].

Maturation of Vaccine Cells by Receptor/Ligand Interaction

CD40-Stimulated Leukemia Cells as Vaccine Cells

As an alternative to the gene-transfer approach, one may also take advantage of the CD40L-induced bystander effect and culture the leukemic blasts ex vivo on CD40L-expressing feeder cells or in the presence of the CD40L

trimer to induce maturation. In ALL, such CD40-stimulated blasts express high levels of MHC class-I and II molecules, show upregulation of ICAM-1 and LFA-3 expression as well as of CD80, CD86 [58] and CD70, a co-stimulatory molecule that also contributes to T-cell activation [127]. These CD40-activated ALL cells serve to generate anti-leukemic T-cell lines in vitro [58, 128]. When combined with IL-4, CD40-activated ALL blasts express the typical DC marker CD83 [48, 60] and stimulate naive T cells to secrete Th1-type cytokines. In addition to acute and chronic B-cell leukemia [58, 59], CD40-induced maturation has also been successfully employed in AML [50], enhancing the T-cell-stimulatory capacity of myelogenous blasts.

Maturation of Leukemic Cells to Dendritic-Like Cells

When AML or CML cells are cultured in the presence of GM-CSF in combination with either IL-4 and TNF-α, or IFN-α, myeloid blasts take on DC-like features with upregulation of the markers CD1a and CD83, MHC and co-stimulatory molecules [48, 51, 52, 54, 129, 130]. In patients with CML, the combined administration of GM-CSF and IFN-α led to in vivo differentiation of mononuclear cells to DCs [131, 132]. In these CML-derived DCs, the bcr-abl fusion products are detectable and stimulation of cytotoxic T-cell activity against parental CML has been demonstrated with low reactivity to normal bone marrow cells [53]. It has been definitively shown that in CML cells, the bcr-abl fusion protein is processed, MHC-binding peptides are generated and presented such that CML cells can serve as target cells to bcr-abl-specific T-cell clones [133]. In a clinical phase-I/II study, reinfusion of CML-derived DCs also led to priming of CML-reactive T cells in vivo and these T cells responded to subsequent in vitro challenge with autologous CML cells [56]. Similarly, DCs generated from non-malignant monocytes and pulsed with different bcr-abl peptides [134, 135] stimulate a potent CML-specific T-cell response [8, 9, 136, 137].

DC Vaccines

Thus, instead of improving the antigen-presenting capacity of leukemic cells themselves, DCs derived from non-malignant precursors may be employed as professional APCs for stimulation of an anti-neoplastic immune response [138]. Due to high levels of MHC, co-stimulatory and adhesion molecule expression, DCs are able to activate unprimed T cells and are therefore ideal tools for vaccine generation [61, 62]. Physiologically, human DCs are localized in tissues and lymphoid organs while in the peripheral blood, they are detectable at a very low frequency of less than 0.5%. In the last years, several protocols have been developed for large scale ex vivo generation of DCs either from CD14+ monocytes [63, 139, 140] or from CD34+ hematopoietic precursor cells [65, 66, 141]. Monocyte-derived DCs are cultured in the presence of GM-CSF and IL-4, while for generation of DCs derived from CD34+ cells additional cytokines such as flt-3 ligand, IL-3 or stem cell factor are employed that allow for initial expansion of myeloid progenitors. Immature DCs employ a number of mechanisms to facilitate antigen uptake such as macropinocytosis [142] or receptor-mediated endocytosis via the C-type lectin receptor DEC205 [143] or the FC receptors CD32 and CD64 [142, 144]. Additional maturation of DCs is required for effective antigen presentation. Ex vivo maturation can be achieved by addition of cytokines such as TNF-α, PEG2 and IL-1β. In the presence of these cytokines DCs upregulate MHC and co-stimulatory molecules that are essential for effective T-cell stimulation [145–147]. In solid tumor patients, DC vaccines derived from CD14+ [148–156] and CD34+ [157–159] cells have been used in clinical phase-I/II studies for stimulation of an anti-neoplastic immune response.

For loading of DCs with tumor antigen, several strategies can be pursued. If tumor-associated antigens (TAAs) have been identified, defined TAA-derived peptides matching the respective MHC-restriction elements may be used for DC loading and subsequent presentation of tumor-specific antigens to the immune system [160]. These TAA-derived peptides can be synthesized under good manufacturing practice conditions to high quantities and have the advantage that once synthesized they are readily available for clinical grade vaccine preparation. Clinical phase-I/II studies principally document the immunological and clinical efficacy of this approach in patients with melanoma, prostate and ovarian cancer [64, 148, 149, 156–159, 161–163].

Leukemia-Specific Targets

Principally, leukemias express an array of antigens that may serve as potential targets for an anti-leukemic immune response. For vaccine generation, the choice of suitable target antigens depends not only on their exclusive or preferential expression in malignant cells but also on their immunogenicity.

As discussed above, in leukemia translocation products, such as the BCR-ABL protein in CML [9, 134, 135], PML-RARα in acute pro-myelocytic leukemia [11], DEK-CAN in AML [12] and ETV6-AML1 in pre-B ALL [10],

are a potential source of target antigens. In patients with multiple myeloma and follicular B-cell lymphoma, peptides derived from the hypervariable region of the immunoglobulin protein called the idiotype have been employed as target antigens. Administration of idiotype-loaded DCs in these patients induces idiotype-specific T-cell responses [155, 159, 164–168] as well as clinical responses in some cases. Whether the applicability of this approach also extends to other B-cell malignancies needs to be evaluated. Another group of potential target antigens are not truly leukemia-specific but are expressed to high levels in leukemic cells. In AML and ALL, HLA-A2-binding peptides derived from the Wilms tumor gene-encoded transcription factor WT-1 [169, 170] stimulate a specific anti-leukemic cytotoxic T-cell response [171–173]. Similarly, DCs loaded with two HLA-A2-binding peptides from the MUC1 protein, an epithelial mucin that is overexpressed in AML and multiple myeloma [174], facilitate the generation of anti-neoplastic cytotoxic T lymphocytes [175]. Proteinase-3 (PR3), a neutrophilic granule protein, is expressed at high levels in AML and CML [176] with negligible expression in normal hematopoietic progenitors. Several HLA-binding PR3 peptides have been identified that elicit a cytotoxic anti-leukemic T-cell response in vitro [177]. In CML patients, detection of cytotoxic PR3-specific T cells in the peripheral blood correlates with clinical responses after bone marrow transplantation or IFN-α treatment [14]. Also clinically, a PR3 peptide vaccine effectively stimulates specific immune responses [178]. The group of so-called 'shared' antigens comprises TAAs that are generally not detected in normal tissue but are expressed in various neoplastic diseases including leukemia. The human telomerase reverse transcriptase (hTERT) for instance is expressed in 85% of cancers as well as in leukemias [179, 180], and HLA-A2- or A24-binding hTERT-derived peptides can be employed to elicit cytotoxic anti-leukemic T-cell responses [15, 16, 181]. Similarly survivin, an apoptosis inhibitor [182], is expressed in different human malignancies including ALL, AML and CML blastic crises but not in normal adult tissue [17, 18]. Survivin-derived MHC class-I-binding peptides can be used for the generation of survivin-specific T cells with anti-leukemic cytotoxic activity [183].

Identification of peptides derived from leukemia-specific or overexpressed antigens as potential T-cell targets facilitates the use of these peptides in clinical DC vaccine applications. While exclusive presentation of a defined antigen minimizes the risk of autoimmune phenomena induced by contaminating self-antigen, peptide-based vaccines may not target a broad enough spectrum of antigens allowing escape mutants to develop. Thus, combination of peptides derived from different antigens generating a 'polyvalent' vaccine may prove more beneficial. Yet, peptide-based vaccines are restricted to malignancies for which specific target antigens have been identified and are only applicable to patients who carry the relevant MHC restriction elements. Several investigators therefore employ lysates of the neoplastic cells as the antigen source. In clinical studies utilizing such lysate-pulsed DCs as the vaccine, induction of T-cell-mediated anti-tumor responses and partial clinical responses in the absence of autoimmune side effects has been demonstrated [148, 150–154, 184, 185]. While for solid tumors DC vaccines have been extensively examined, in hematological malignancies, with the exception of myeloma and CML, clinical experience with this vaccine approach is currently limited.

Thus while the spectrum of available vaccines for malignant hematological diseases is broad ranging from genetically modified leukemic cells to DCs, careful evaluation in well-controlled clinical studies of both immune and clinical responses is required to identify the optimal vaccination strategy for each of the different leukemic disease entities.

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Author Index Vol. 110, No. 2–3, 2003

Acta Hæmatologica

Ackermann, B. 160
Bardenheuer, W. 93
Basso, L. 121
Baum, C. 107
Bollard, C.M. 139
Bostrom, N. 121
Brenner, M.K. 154
Burger, S.R. 121
Chen, X. 110
d'Azzo, A. 71
Dilloo, D. 59, 160
Dinauer, M.C. 86
Engel, B.C. 60
Flasshove, M. 93
Glouchkova, L. 160
Goebel, W.S. 86
Guenther, W. 110
Heslop, H.E. 139, 149
Huls, M.H. 149
Kadidlo, D.M. 121
Kalle, C. von 107
Kohn, D.B. 60
Kolb, H.-J. 110
Kramm, C.M. 132
Ledderose, G. 110
Moritz, T. 93
Orchard, P.J. 121
Rooney, C.M. 139, 149
Roskrow, M. 110
Rössig, C. 154
Savoldo, B. 139
Schleuning, M. 110
Schmid, C. 110
Seeber, S. 93
Weber, M. 110
Woiciechowski, A. 110

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Subject Index Vol. 110, No. 2–3, 2003

Adeno-associated virus 78
Adenosine deaminase deficiency 61
Adenoviral vectors 78, 161
Adoptive immunotherapy 110, 139, 149, 154
Allogeneic T lymphocytes 121
Animal models 71, 89, 111, 163
Cell therapy 121
Chimeric receptors 65, 154
Chimerism 110
Chronic granulomatous disease 86
Clinical gene transfer studies 61, 77, 98
Costimulation 115, 154, 163
Cytotoxic T lymphocytes 149, 154
Dendritic cells 116, 160
Donor lymphocyte transfusion 110, 132
Drug resistance 93
Epstein-Barr virus 139, 149
Gene therapy 60, 71, 86, 93, 107, 132, 154
– transfer 76, 160
Graft-versus-host disease 115, 121, 132
Graft-versus-leukemia effect 115, 121, 132
Hematopoietic stem cells 60, 86, 93
Herpesviral vectors 161
Hodgkin's disease 114, 149
HSV-tk 121
Immunodeficiency 60
Lentiviral vectors 77, 136
Leukemia 112, 132, 160
Lysosomal enzymes 71
– storage diseases 71
Mutagenesis 62, 107, 135
NADPH oxidase 86
Phagocytes 86
Post-transplant lymphoproliferative disease 139
Retroviral vector 61, 76, 88, 93, 107, 123, 136
Safety aspects, gene transfer 101, 107, 135
Stem cell transplantation 110, 121
Suicide gene therapy 121, 132
T cell receptor 65, 154
Transformation 62, 107, 135
Transplantation 139
– tolerance 110
Vaccines 160
Viral vectors 71

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