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GENETIC LEVEL THERAPY FOR NEONATAL PROBLEMS

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ABSTRACT

In spite of developments of neonatal intensive care medicine, it is still difficult or impossible to treat several inherited genetic disorders using conventional pharmacological methods. Gene therapy is a promising alternate approach for treating a variety of genetic disorders. By the time the patient reaches adulthood, however, it is often too late for effective treatment. But in several of these cases, neonatal gene therapy appears potentially useful against inherited disorders that are not obviously treatable through any other methods. Application of gene therapy to treat genetic and infectious diseases may have several advantages if performed in newborns. Because of the minimal adverse effect of the underlying disease on cells of the newborn, the relatively small size of infants, and the large amount of future growth, gene therapy may be more successful in newborns than in older children or adults. The presence of umbilical cord blood from newborns provides a unique and susceptible target for the genetic modification of hematopoietic stem cells. In our first trial of gene therapy in newborns, we inserted a normal adenosine deaminase gene into umbilical cord blood cells of three neonates with a congenital inunune deficiency. The trial demonstrated the successful transduction and engraftment of stem cells, which continue to contribute to leukocyte production more than 3 years later. A similar approach may be taken to insert genes that inhibit replication of H1V-l into umbifical cord blood cells of H1V-1-infected neonates. Many other metabolic and infectious disorders could be treated by gene therapy during the neonatal period if prenatal diagnoses are made and the appropriate techmcal and regulatory requirements have been met. This review describes the strategy for neonatal gene therapy for inherited disorders and presents preclinical neonatal gene therapy.

KEYWORDS: Neonatal gene therapy, Metachromatic, leukodystrophy, hypophosphatasia.

INTRODUCTION

Although there have been significant advances in neonatal intensive care medicine, several neonatal disorders remain major causes of mortality and morbidity. Consequently, there is an urgent need for development of new safe and effective therapies to improve the outcomes of these intractable and devastating neonatal disorders. Gene therapy is an exciting and promising approach to treat many diseases for which there are still no effective therapies. To date, more than 2400 clinical trials of gene therapy protocols have been attempted in effort to treat various genetic diseases as well as many types of cancers and infectious diseases. The results of preclinical studies suggest that neonatal gene therapies represent potentially effective treatments for currently intractable neonatal disorders. However, although neonatal gene therapies have several advantages over similar therapies used in adult patients, there is as yet no clinical protocol for use of gene therapy in newborn infants. This chapter describes a strategy for the use of neonatal gene therapy in the treatment of inherited disorders and presents preclinical neonatal gene therapy data for two inherited disorders, metachromatic leukodystrophy (MLD) and hypophosphatasia (HPP). We also discuss the utility, advantages, problems and the potential of neonatal gene therapeutic approaches for the treatment of inherited disorders.[1 - 6]

Advantages of gene therapy in newborns

The neonatal period may provide an optimal time for gene therapy. By intervening against dis- eases early in life, it may be possible to completely prevent the development of symptoms. For example, in the lysosomal storage diseases, subjects are essentially nonnal at birth.[7] Maternal enzyme can act in the fetus to eliminate substrate accumulation during prenatal life. After birth, substrate accumulation begins and the signs and symptoms of the disease progressively develop over the first few years of life. Effective gene therapy shortly after birth would prevent the development of clinical problems. Complications of the disease that develop later

in life may hinder the effectiveness of gene therapy. For example, the accumulation of abnormal, thick mucus in the bronchial tree of patients with cystic fibrosis (CF)2 may hinder gene delivery by a transbronchial approach. Newborns with CF have not yet suffered repeated episodes of infection, and thus their airways may be more conducive to gene dispersion. The relatively smaller size of neonates also makes the logistics of performing gene therapy simpler, since the absolute amount of gene vector needed is likely to be significantly less than would be needed to treat children or adults. Also, the large amount of growth a newborn will ultimately undergo may result in an am- plification of the genetically modified cells.

- May prevent development of signs and symptoms of some diseases.
- Disease manifestations may not be present to interfere with gene delivery.
- Many tissues have higher percentage of proliferating cells, increasing transduction frequency by retroviral vectors.
- Small body sizes decreases vector needs. Rapid future growth amplifies transduced cell numbers.

Systemic gene transfer to neonates has several advantages over treatment of the adults. First, as mentioned above, neonatal gene therapy has the potential to overcome the limitation imposed by the BBB on treating genetic disorders of the CNS. Because the BBB is developmentally immature during the perinatal period, AAV-mediated neonatal gene therapy is a highly promising strategy for treating genetic neurological diseases. Second, because the immune system is immature, neonates are immunologically tolerant of the transgene and/or viral vector. Immune rejection of the transgene product by neutralizing antibodies is a severe problem for gene therapy in adults. Third, treatment administered soon after birth may enable prevention of early-onset genetic disease. Finally, neonates can be effectively treated with a smaller amount of viral vector than adults. Using smaller amounts of viral vector is superior with respect to both safety and cost. Taken together, these advantages make systemic neonatal gene therapy a promising method for treating systemic genetic diseases.[8-10]

Umbiucal cord blood cells for gene therapy of newborns

Some unique aspects of neonatal physiology may make the newborn infant a more effective target for gene therapy. A prime example is the hematopoietic stem cells present in umbilical cord blood. Hematopoietic stem cells, i.e., cells capable of giving rise to the entire spectrum of mature hematopoietic and lymphoid cells, are the target of gene therapy for a variety of hematologic and immunologic disorders.[11] However, the vast majority of stem cells in adult bone marrow is in a quiescent, noncycling state and therefore is not susceptible to transduction with Moloney munne leukemia virus-based retroviral vectors. The umbilical cord blood of neonates provides a unique alternative to bone marrow as a source of hematopoietic stem cells. At birth, a large portion of the neonatal blood volume is circulating through the vessels of the placenta for oxygen and nutrient exchange with the maternal circulation. After clamping of the umbilical cord, this portion of the neonatal hematopoietic cells remains in the placenta and typically is discarded. Studies over the past decade have shown that um- bilical cord blood can be collected from the placenta and used as an alternative source of hematopoietic stem cells for allogeneic transplantation.[12,13] Indeed, the use of umbilical cord blood for transplantation has been growing rapidly. Although the relative merits of allogeneic umbilical cord blood compared to allogeneic bone marrow as a source of hematopoietic stem cells are not fully determined, initial results indicate that umbilical cord blood causes a lower rate of graft vs. host disease. Of direct relevance to gene therapy, data suggest that a larger fraction of putative stem cells in umbilical cord blood are in active cycle than those found in adult bone marrow.[14] Thus, retroviral vector-mediated gene transfer into umbilical cord blood cells may yield a higher percentage of transduced stem cells than with bone marrow. Most considerations of performing gene therapy using hematopoietic stem cells (either from bone marrow or umbilical cord blood) have focused on the correction of autologous cells. In fact, the ability to perform transplantation with a patient's own cells is the key theoretic advantage of gene therapy over allogeneic bone marrow transplant, avoiding the immunologic problems of graft rejection and graft vs. host disease. However, there are some settings where gene transduction of allogeneic cells may also be useful. Cells could be engineered by gene insertion to overexpress therapeutic proteins, such as coagulation factors, lysosomal enzymes, adenosine deaminase, etc., or to be resistant to either infectious agents or chemotherapy-induced myelosuppression. Here, the higher transduction efficiency and lower incidence of graft vs. host dis- ease mediated by umbilical cord blood may make it the preferred cell target over bone marrow. This approach could then be used with children and adults who are not likely to have access to their own umbilical cord blood. Other neonatal tissues may also have a greater proliferative fraction than their childhood and adult counterpart, thus allowing for more effective gene therapy. Among the organs that undergo significant postnatal growth involving cellular proliferation are the liver, lungs, and central nervous system.

Adeno-associated virus-mediated gene transfer to neonate

The first application of gene therapy for newborns was performed by our group at Childrens Hospital Los Angeles in 1993.[15] Three infants were diagnosed prenatally as having adenosine deaminase (ADA) deficiency, a genetic cause of severe com- bined immune deficiency (SCID). All three children came from families with children who had already been affected; therefore,

amniocenteses were performed during the pregnancy and identified ADA deficiency. The prenatal diagnosis allowed implementation of gene therapy in the neonatal period for these patients. In the 3 months that elapsed between the diagnoses and the birth of the infants, the first clinical gene therapy protocol, which involved treatment of ADA deficiency by gene insertion into peripheral blood T lymphocytes by Drs. Blaese, Anderson, and Culver,[16] was amended for the use of umbilical cord blood cells as the target for gene transfer. Approval was obtained from the relevant institutional review boards, the National Institutes of Health (NIH) Recombinant DNA Advisory Committee, and the Food and Drug Administration to use the same retroviral vector supernatant preparations that had been certified for use in the NIH trial of T cell-directed gene therapy. The vector used was the LASN vector constructed by Dr. Dusty Miller at the Fred Hutchinson Cancer Research Center.[17] LASN carries a normal human ADA cDNA under transcriptional control of the Molony murine leukemia virus LTR, as well as the bacterial neomycin resistance gene under control of an SV4O promoter. At birth, umbilical cord blood was collected from each of the three infants. Between 3 and 18 million cells bearing the CD34 stem/progenitor cell antigen were isolated using the CellPro Ceprate column. The CD34 cells were incubated for 3 days in medium supplemented with the growth factors interleukin-3, interleukin-6, and c-kit ligand (stem cell factor). LASN retroviral vector supernatant was added on days 1, 2, and 3 of culture. At the end of culture, when it had been determined that the samples were free of microbial contamination, the cells were extensively washed and infused intravenously into each neonate on day 4 of life. In vitro analyses of the transduced cell showed that between 12 and 20% of the CD34 cells that grew as CFU-GM colonies were G418-resistant. The infants were started on enzyme replacement therapy within a few days of birth, with twice-weekly intramuscular injections of polyethylene glycol-conjugated bovine adenosine deaminase (PEG-ADA). Within a few weeks of the institution of PEG-ADA therapy, the patients' lymphopenia improved and they developed blastogenic responses to the nitrogen, phytohemagglutinin (PHA).

The infants were immunized with tetanus toxoid at approximately 6 months of age and developed tetanus-specific blastogenic responses. To the present time (3 years after birth), the patients have continued to receive PEGADA therapy and have shown normal growth and development. The major measurement of gene transduction of hematopoietic stem cells has been analysis of peripheral blood leukocytes to determine the frequency of cells containing the LASN vector. We have observed the stable presence of mature granulocytes, monocytes, and B and T lymphocytes containing the gene. However, the frequency of gene-containing cells has been quite low, in the range of 1 gene-containing cell per 3,000-10,000. This frequency is in accord with theoretic expectations from a low percentage of stem cells transduced, followed by further dilution from the endogenous, nonablated marrow. The continued presence of these cells for more than 3 years after a single transplantation demonstrates that some of the cells transduced from the neonatal umbilical cord blood are long-lived stem cells capable of sustained hematopoiesis.

It will be necessary in future studies to determine whether there is a higher rate of transduction and engraftment using umbilical cord blood from neonates vs. what is achieved in children or adults using bone marrow or mobilized peripheral blood stem cells. ADA-deficient SCID has been one of the primary disease candidates for gene therapy because it is ex- pected that genetically corrected T lymphocytes would have a selective survival advantage and accu- mulate to levels that might be therapeutic. This ex- pectation was based on previous observations of patients with SCID who underwent allogeneic bone marrow transplant and in whom the engraftment of undetectable numbers of donor marrow cells re- sulted in the development of a complete immune system.[18] Recently, two reports have documented cases of spontaneous remission of SCID (one due to ADA deficiency and one of the X-linked form) due to the reversion to normal of the disease-causing mutations.[19,20] From these cases, it appears that even a single genetically normal T lymphoid precur- sor can produce sufficient numbers of T lymphocytes under the selective pressure of these diseases to re- store normal immunity. It is likely that exogenous enzyme replacement therapy may blunt the selective advantage that the progeny of genetically corrected hematopoietic stem cells expressing ADA would have. Therefore, approximately 1 years after the gene therapy was performed, the dosages of PEG-ADA received by all the patients were reduced. Subsequent to the dosage re- duction, we have seen a marked increase in the frequency of gene-containing T lymphocytes to 1-10%. The frequencies of transduced cells in the other hematopoietic lineages have not increased, since they do not have a selective advantage from expressing ADA. The patients are still on reduced dosages of enzyme replacement therapy; therefore, it is not possible to say whether they have received any immunologic benefit from the gene transduction. However, the selective advantage of genetically corrected cells in SCID patients has been clearly demonstrated.[21] It is likely that a similar selective advantage would be seen for genetically corrected cells in patients with other forms of SCID as well as Wiskott-Aldrich syndrome, X-linked agammaglobulinemia, and Fanconi's anemia. All of these diseases should be prime targets for gene therapy when the relevant genes are cloned and their biology is sufficiently understood.

Gene therapy for hilt-i infection in newborns

Whereas the initial diseases considered for gene therapy were inherited monogenic disorders, more re- cently attention has turned to applying gene therapy to common noninherited diseases. One condition that has received

considerable attention is HIV-1 infection, the cause of AIDS.[22,23] A variety of synthetic genes (e.g., antisense, ribozymes, dominant negative mutants, intracellular antibodies) have been developed that are capable of rendering cells resistant to HIV-1 infection and/or replication. Theoretically, inserting these genes into a patient's hematopoietic and lymphoid cells could result in the development of an immune system resistant to the cytopathic effects of HW-1. Our group and others.[24,25] have shown that insertion of these "antiHIV-1" genes into CD34 hematopoietic cells leads to resistance to HIV-1 replication in the monocytic cells grown from the progenitor cells. Clinical trials are being initiated to examine the effects of genetically modifying hematopoietic stem cells to become resistant to HIV-1. Although most initial studies will use either bone marrow or G-CSF-mobilized peripheral blood from adults or older children, it is possible that gene therapy could be instituted using umbilical cord blood from HIV-in fected neonates. The use of cord blood from HIV-1-infected neonates has potential benefits in addition to the more effective transduction of um- bilical cord blood vs. bone marrow. Since most HIV1 transmission occurs perinatally, the umbilical cord blood cells should contain a sufficient number of normal hematopoietic stem cells for gene transduction, which may be diminished in the bone marrow of HIV-1-infected children and adults.[26] The development of mature, functional T lymphocytes from transduced stem cells is likely to require normal thymic function. It is known that thymic function is greatest in newborns and declines with age, so that by the second decade of life thymic function is significantly reduced.[27,28] Also, HIV-1 infection itself has been reported to degrade thymic function. Therefore, stem cell-directed gene therapy in HIV-1- infected newborns is likely to have the greatest possibility of producing a broad T lymphocyte repertoire because of the presence of a young, functional thymus. One logistic difficulty in using umbilical cord blood to treat HIV-1-infected children is the unpredictable likelihood of HIV-1 infection developing in an infant born to an HIV-1-infected mother. Landmark studies have shown that the rate of maternal to infant HIV transmission can be reduced to as low as 10% by treating the mother with antiretroviral agents.[29] It would not be reasonable to perform gene therapy in all infants born to HIV-1-infected mothers if only 1 of 10 is actually infected. Observation for 1 to 3 months after birth is required before it can be determined whether an infant has become infected with HIV-1. Thus, to perform a clinical trial of gene therapy, the umbilical cord blood needs to be collected from infants born to FIIV-1-infected mothers and cryopreserved until the infection status of the infant can be definitively assessed. When an infant is documented to be infected by HIV-1, their own cord blood specimen can be thawed, transduced, and infused. This approach necessitates the establishment of umbilical cord blood banks for cryopreservation of blood from a large group of HIV-1- exposed infants.

Among the numerous viral and nonviral vectors that have been developed to deliver genes of interest into target cells, adeno-associated virus (AAV) vector has emerged as a particularly promising tool for gene delivery, thanks to its safety (AAV is not pathogenic) and its ability to transduce nondividing cells.[30] AAV vector serotypes (mainly 1–12), depending on the target.[31] results after intravenous injection into neonatal mice of AAV vector serotypes, harboring the luciferase gene. Expression of luciferase is detected within 3 days and continued for more than 16 weeks with no decrease in expression.[32] Serotype 9 mediated the highest expression during the observation period. In addition, using an AAV vector encoding green fluorescent protein (GFP), determined that the organs most efficiently transduced are the liver, heart and muscle.[33] Moreover, although transduction efficiency was not as high, the central nervous system (CNS) was also transduced after intravenous injection of AAV vector, which apparently passes through the blood-brain barrier (BBB).[34] in neonatal mice.[35] Thus, a systemically administered AAV vector is able to transduce several important target organs in neonatal mice, including the CNS, and mediate expression of a gene of interest for a prolonged period of time.[36-38]

Neonatal gene therapy for metachromatic leukodystrophy

Metachromatic leukodystrophy is an inherited, autosomal recessive lysosomal storage disease (LSD) caused by a deficiency in the lysosomal enzyme arylsulfatase A (ASA), which catalyzes the degradation of galactosyl-3-sulfate ceramide (sulfatide (Sulf)), a major myelin sphingolipid.[39] This disease is characterized by myelin degeneration, mainly in the CNS, and clinically by progressive motor and mental deterioration that is ultimately lethal. Therefore, the major target organ for treatment of this disease is the CNS, and the aim is to arrest or reverse the progression of the neurological symptoms. A major obstacle, however, is the BBB, which limits delivery of systemically administered therapeutic molecules to the brain.[40] It is therefore hoped that systemic administration of an AAV vector harboring ASA during the neonatal period would be useful for treating the CNS. We previously showed that a single systemic injection of AAV vector encoding human ASA (AAV/hASA) into neonatal ASA knockout (MLD) mice results in the wide distribution of ASA in the brain and correction of the biochemical and neurological phenotypes.[41] A single systemic injection of AAV/hASA enables transduction of the CNS in neonates but not in adults. Efficient hASA expression was detected in the brain of AAV/hASA treated at the neonatal period of MLD mice. PCR analysis confirmed that AAV vector genome was observed only in neonatal-treated MLD mice. Moreover, sustained expression of hASA in plasma was detected for at least 30 weeks after intravenous injection into neonatal MLD mice, while only transient increase in plasma hASA was obtained when injected into either adult MLD

mice or wild-type C57Bl/6 mice. Vector injection into adult NOD-SCID mice led to sustained secretion of hASA into the circulation, suggesting that immune responses to hASA are a major hurdle for successful gene therapy in immunocompetent adult MLD mice. It thus appears that the systemic injection of AAV vector during the neonatal period is a potentially useful means of treating neurological disorders.

Neonatal gene therapy for hypophosphatasia

Hypophosphatasia is an inherited disease caused by a deficiency of tissue-nonspecific alkaline phosphatase (TNALP).[42] The major symptom of human HPP is hypomineralization, rickets or osteomalacia, although the clinical severity is highly variable. Patients with infantile HPP may appear normal at birth but gradually develop rickets before reaching 6 months of age. Neonatal gene therapy is a promising strategy for treating infantile HPP by preventing early onset. We have shown that the phenotype of TNALP knockout mice,[43] which mimics the severe infantile form of HPP, can be prevented by a single neonatal injection of AAV vector encoding bone-targeted TNALP in which a deca-aspartate tail is linked to the C-terminus of soluble TNALP (AAV/TNALP-D10). Sustained expression of TNALP and phenotypic correction of TNALP knockout mice were observed following the neonatal gene therapy.[44] X-ray analysis showed that treated TNALP knockout mice grow as well as normal wild-type mice.

Problems of neonatal gene therapy

There are several problems that must be overcome before neonatal gene therapies can be used in humans. First, safety concern must be addressed, as there is the possibility of tumor development and of germ-line transmission. It was reported that liver and lung cancers appeared in some mice treated using AAV-mediated neonatal gene therapy.[45,46] In addition, differences in developmental stages of organs in mice and humans may be another problem. The immune system in mice is less mature at birth than that in larger animals, and the human BBB is functionally mature before birth. It is therefore not clear whether the same beneficial effect of neonatal gene therapy seen in mice would be achieved in human infants. These problems must be overcome before there can be clinical trials of neonatal gene therapy.[47,48]

Summary and future developments

We have shown that AAV-mediated gene transfer in neonatal mice has characteristics that could potentially overcome the problems encountered with current gene therapy protocols. Although no active trials have been planned, other metabolic disorders exist in which early intervention is likely to provide benefits for the subjects including urea cycle defects, lysosomal storage diseases, cystic fibrosis, muscular dystrophy, and other neurodegenerative disorders. As the technology advances for more effective gene therapy, it is likely that these diseases will be targeted in the neonatal period. It re- mains to be seen whether gene therapy will provide better treatments for these diseases than those that currently exist. However, before applying neonatal gene transfer to humans, several important issues must be addressed. In particular, the safety of neonatal gene transfer must be carefully evaluated using large animal models, including nonhuman primates. Nonetheless, because of its advantages over gene therapies used to treat genetic disorders in adults, safe and effective neonatal gene therapy has the potential to be an invaluable method for treating genetic diseases.

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