Advances in the Treatment of Chronic Granulomatous Disease by Gene Therapy

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Abstract: Gene transfer into hematopoietic stem cells has been successfully used to correct immunodeficiencies affecting the lymphoid compartment. However, similar results have not been reported for diseases affecting myeloid cells, mainly due to low engraftment levels of gene-modified cells observed in unconditioned patients. Here we review the developments leading to a gene therapy approach for the treatment of Chronic Granulomatous Disease (CGD), a primary life threatening immunodeficiency caused by a defect in the oxidative antimicrobial activity of phagocytes. Although the disease can be cured by bone marrow transplantation, this treatment is only available to patients with HLA-identical sibling or matched unrelated donors. One therapeutic option for patients without suitable donor is the genetic modification of autologous hematopoietic stem cells. Although early attempts to correct CGD by gene therapy were unsuccessful, these studies demonstrated the safety and limitations of gene transfer into hematopoietic stem cells (HSC) of CGD patients using retroviral vectors. The recent development of advanced gene transduction protocols together with improved retroviral vectors, combined with low intensity chemotherapy conditioning, allowed partial correction of the granulocytic function with a significant clinical benefit in treated patients. These results may have important implications for future applications of gene therapy in myeloid disorders and inherited diseases using hematopoietic stem cells.

Keywords: Chronic granulomatous disease, immunodeficiency, gene therapy, low intensity conditioning, busulfan, retroviral vectors, hematopoietic stem cells, gp91phox

GENERAL BACKGROUND

Chronic Granulomatous Disease (CGD) is an inherited immunodeficiency of the phagocytic system characterized by life threatening bacterial and fungal infections. In the western world CGD occurs with an overall incidence between 1:200 000 and 1:250 000 life births [Winkelstein et al., 2000]. Severe and recurrent infection episodes lead to hospitalization through life resulting in restrictions in life quality and education [Roos et al., 1999]. Infections and organ dysfunction like chronic inflammatory granulomas, colitis/enteritis or granulomatous obstruction of either the gastric outlet or the urinary tract, and chronic pulmonary restriction are often seen in older CGD patients. Although S. aureus is the most frequent isolated organism overall, the most common cause of death are pneumonitis and/or sepsis due to Aspergillus species or B. cepacia [Winkelstein et al., 2000] with an overall death rate of 2% (autosomal recessive CGD) to 5% (X-linked CGD) per year. The underlying causes of the disease are defects in members of the nicotinamide dinucleotide phosphate (NADPH) oxidase complex resulting in a deficient antimicrobial activity of phagocytes (Fig. 1). The NADPH oxidase plays an important role in microbial killing by reducing molecular oxygen to superoxide which subsequently reacts to form reactive oxygen species (ROS) like hydrogen peroxide, hypochlorous acid and hydroxyl radicals. The generation of ROS is accompanied by potassium influx and pH changes within the phagocytic vacuoles leading to the activation of neutrophil proteases (neutrophil elastase, cathepsin G) which are also essential for the antimicrobial activity of phagocytes [Segal et al., 2005].

The NADPH oxidase enzyme complex consists of two membrane-bound subunits, gp91phox and p22phox, as well as three cytosolic components p47phox, p67phox and p40phox [Roos et al., 2003; Cross et al., 2004]. In addition the low-molecular-weight GTP-binding proteins Rac1, Rac2 and Rap1A are involved in the regulation of the NADPH oxidase activity [Abo et al., 1991; Knaus et al., 1991]. Approximately two third of all CGD cases result from mutations within the X-linked gp91phox gene, followed by the autosomal recessive forms of CGD, with p47phox defects accounting for 30% of all CGD cases while only 5% of the cases are due to mutations in p67phox - or p22phox. CGD patients with a defective p40phox subunit have not been reported yet.

STANDARD TREATMENT

Conventional treatment of CGD patients consists of lifelong prophylaxis with antibiotics [Margolis et al., 1990], antimycotics such as itraconazole [Gallin et al., 2003], and/or interferon gamma [Group TICGDCS, 1990; Marciano et al., 2004]. Additionally, during episodes of severe infections the immune system of the patients can be temporarily
supported by allogeneic granulocyte infusions [Tsuge et al., 1998], which are however limited by the risk of antibody formation against foreign antigens [Zhang et al., 2001]. These prophylactic medications have improved health conditions and have helped to reduce death rates [Winkelstein et al., 2000]. Despite these treatments, prophylaxis cannot completely replace the protective function of NADPH oxidase. The annual mortality of CGD patients is still between 2% (autosomal recessive) and 5% (X-linked) [Winkelstein et al., 2000].

**HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)**

One alternative to conventional antibiotic treatment is allogeneic hematopoietic stem cell transplantation (HSCT), presently the only curative therapy for the disease. The majority of patients are X-linked CGD children transplanted with matched family donors. Most patients received an HSCT obtained from sibling donors. An overall treatment related mortality of 15% (4/27) was calculated for all treated patients. Patients, who suffer from active therapy resistant infections and/or organ dysfunction prior to HSCT are classified as late stage disease patients, while those with no organ dysfunction or active infections at the time of transplantation are classified as early stage disease patients. Importantly, HLA-genoidentical HSCT in patients with active inflammation or organ disability due to chronic infection is still feasible, with acceptable survival (75%, 12/16) and increased quality of live [Seger et al., 2002]. Horwitz et al. (2001) treated 10 patients with chronic granulomatous disease with a protocol based on a non ablating conditioning regimen and T-cell depleted transplants. In this study, mortality rate was high up to 30%, because of engraftment failure, graft versus host disease (GvHD) and Pneumonia. The best published treatment results were all achieved with a myeloablative regimen using busulfan and unmodified bone marrow as transplant.

The major risk factors associated with bone marrow transplantation (BMT) are GvHD and inflammatory flare-up at sites of infection [Seger et al., 2002]. If transplantation is delayed to adolescence or later, the chances of invasive fungal infections increase [Hasui M et al., 1999]. The evidence from patients with other forms of primary immune deficiencies suggests that BMT is most successful when done early, as younger patients have less end-organ damage and a lower risk of GvHD. Based on data from the year 2000, a highly compatible unrelated donor is available for 52% of the patients [Tiercy et al., 2002]. The actual numbers should even be higher now as the number of HLA-typed volunteer donors available through the BMDW Registry (www.BMDW.org) reached 10 million worldwide. The present policy therefore aims at transplanting children at a younger age, just when they are developing severe illness, using an HLA identical related or unrelated donor.

Transplantations other than with perfectly matched donors are presently not recommended due to the high morbid-

**Fig. (1).** CGD is caused by defects in the nicotinamide dinucleotide phosphate (NADPH) oxidase complex resulting in a deficient antimicrobial activity of phagocytes. The NADPH oxidase plays an important role in microbial killing by reducing molecular oxygen to superoxide which subsequently reacts to form reactive oxygen species (ROS). The NADPH oxidase enzyme complex consists of two membrane spanning subunits, gp91phox and p22phox, as well as three cytosolic components p47phox, p67phox, p40phox. Approximately two third of all CGD cases results from mutations within the X-linked gp91phox gene, followed by the autosomal recessive forms of CGD, with p47phox defects accounting for 30% of all CGD cases while only 5% of the cases are due to mutations in p67phox or p22phox.
ity and mortality rates associated with this kind of treatment. For those patients without appropriate donor an alternative curative therapy is highly desired.

GENE THERAPY

As a monogenetic disease CGD should be amenable to gene therapy. The genes encoding for the subunits of the NADPH oxidase have been cloned. Several assays are currently available to estimate functional correction of affected cells (for recent review see [Lambeth et al., 2004]). Moreover, data from variant forms of CGD and from healthy carriers of X-CGD with 10% normal neutrophils suggest that correction of the disease in a fraction of CGD cells could be sufficient to alleviate the symptoms of the disease [Mills et al., 1980; Woodman et al., 1995; Mardiney et al., 1997; Johnston et al., 1985]. This observation together with a potential synergistic effect between wild type or gene corrected neutrophils and CGD neutrophils in antimicrobial activity [Rex et al., 1990] have motivated the development of a gene therapy protocol for the treatment of CGD. The major draw back of a gene therapy approach for CGD is the lack of survival or proliferative advantage of transduced over non-transduced cells. Therefore bone marrow conditioning or positive selection of transduced cells in vivo will be required in CGD to engraft sufficient numbers of HSC to provide long term correction. Indeed animal studies have shown sustained marking at therapeutically relevant levels in both lymphoid and myeloid lineages after gene therapy, if the protocols were combined with non-myeloablative conditioning [Huhn et al., 1999; Rosenzweig et al., 1999]. Furthermore the pre-treatment of X-CGD mice with only submyeloablative radiation or chemotherapy doses allowed the durable engraftment of substantial numbers of functionally corrected long-term repopulating stem cells [Goebel et al., 2004], suggesting that similar protocols could support engraftment and correction of the disease in humans.

A significant break trough in the field of gene therapy was described in Phase I/II gene therapy trials aimed at the correction of X1-SCID and ADA-SCID [Cavazzana-Calvo et al., 2000; Aiuti et al., 2002; Gaspar et al., 2004]. These trials provided definitive proof that gene therapy is an alternative for the correction of immunodeficiencies, but have also revealed the limitations and risks associated with this novel technology [Hacein-Bey-Abina et al., 2003]. For CGD the results obtained in the ADA-SCID trial were particular relevant since in this study bone marrow conditioning was used for the first time to improve engraftment and survival of gene transduced cells [Aiuti et al., 2002]. The first patients described by Aiuti and colleagues were treated with 2 mg/kg/day busulfan for 2 days before reinfusion of the transduced autologous CD34+ cells. Meanwhile 10 patients have been treated with this protocol. Most (but not all) ADA-SCID patients have shown sustained engraftment of gene transduced cells and increased lymphocyte counts, leading to improved immune functions and clinical status. Most relevant to CGD was the observation, that with this kind of treatment a substantial amount of gene marking (~10%) could be achieved in myeloid cells depending on the level of myelosuppression achieved in each of the patients.

GENE THERAPY TRIALS FOR CGD

The first Phase I/II gene therapy trial for CGD was carried out by Malech and colleagues at the NIH back in 1995. In this study 5 adult patients with a p47phox defect were treated with G-CSF mobilized peripheral blood CD34+ cells after genetic modification of the cells with a p47phox expressing retroviral vector [Malech et al., 1997]. After transduction, CD34+ cells were reinfused into the patients without marrow conditioning. Although the level of functionally corrected granulocytes after gene transfer was high in vitro (27-90%), the percentage of functionally corrected granulocytes in vivo ranged between 0.004 to 0.05% of total peripheral blood granulocytes and persisted at this level for up to 6 months after reinfusion. The same group initiated a second Phase I trial in 1998. In this trial, 5 patients with X-linked CGD, were treated with gene modified peripheral CD34+ cells. Several modifications were included in this trial: CD34+ cell mobilization was done with Flt3-Ligand (50µg/kg) and GM-CSF (5 µg/kg) and retroviral transduction was done on 4 subsequent days resulting in a transduction efficiency in colony forming cells of 48% - 89%. Moreover patients received the genetically modified cells in two cycles 50 days apart without bone marrow conditioning. Despite these modifications the level of functionally corrected cells (DHR positive cells) remained low and was detected at a range between 0.2% and 0.6% 3-4 weeks after the second reinfusion and remained at this level for the next 4 to 6 months [Malech et al., 1998].

A third trial was conducted by Mary C. Dinauer and colleagues at Indiana University using an MSCV-based bicistronic retroviral vector containing the gp91phox and the neomycin resistance genes. 2 adults with X-CGD were treated with gene modified cells in the absence of bone marrow conditioning. Superoxide production was detected in both patients in 0.1% of peripheral blood neutrophils and persisted for almost 9 months after infusion [Barese et al., 2004].

Although the level of correction achieved in these trials was low, they provided clear evidence that a gene therapy approach for CGD is feasible and paved the way for further clinical trials.

In 2002 our group started the treatment of an X-CGD patient with gene modified CD34+ cells using a mild myelosuppressive conditioning regimen (cyclophosphamide 1g/m /day on 4 consecutive days) prior to reinfusion of transduced cells. In this trial we used a bicistronic vector containing the gp91phox cDNA and a marker gene (dLNFR). The vector was produced from PG13 packaging cell lines at a titer of 1x10^6 /ml. In vivo transduction of 1g/kg and GM-CSF (5µg/kg) and retroviral transduction was done on 4 consecutive days resulting in a transduction efficiency in colony forming cells of 48% - 89%. Moreover patients received the genetically modified cells in two cycles 50 days apart without bone marrow conditioning. Superoxide production was detected in both patients in 0.1% of peripheral blood neutrophils and persisted for almost 9 months after infusion [Barese et al., 2004].

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of gene transduced cells. Alternatively, the transduction protocol used at that time may have impaired the survival of real multipotent repopulating stem cells.

To improve the quality and engraftment of gene transduced cells several modifications in vector design and transduction procedures were introduced into a new gene therapy trial conducted in Frankfurt (Fig. 2). Two adult X-CGD patients were treated in Frankfurt in 2004. P1 (26 years old) was first diagnosed at the age of 3. Molecular analysis revealed that he had a point mutation in exon 9 of the CYBB gene leading to the amino acid substitution T343P within the binding domain of FAD in the gp91phox protein. P2 (25 years old) was found to have X-CGD at the age of 1. He was found to have a 5 bp deletion in exon 10 leading to a stop codon at amino acid position 501 within the NADPH binding site of gp91phox. Both patients were classified as X-CGD X0 due to the complete absence of gp91phox protein.

Peripheral blood CD34+ cells were obtained from both patients after G-CSF mobilization. The total number of cells collected was 1.6x10⁸ and 3.8x10⁸ for P1 and P2, respectively. The CD34+ cells were cultured for 36 hours at a concentration of 10⁶ cells/ml in the presence of Flt3-L (300 ng/ml), SCF (300 ng/ml), TPO (100 ng/ml) and IL3 (60 ng/ml) in the absence of serum. Thereafter cells were transduced for 24 hours by incubating the cells with retroviral particles bound to Retronectin. This procedure was repeated 3 times. The vector used was a monocistronic gammaretroviral vector encoding gp91phox. Transduction efficiency ranged between 45.2% (P1) and 39.5% (P2) as measured by the expression of gp91phox on CD34+ cells. Vector copy per transduced cell was derived from a quantitative PCR and found to be 2.6 for P1 and 1.5 for P2. P1 received 5.1x10⁶ CD34+/gp91phox+ cells per kg while this number was 3.6x10⁶ for P2. Before reinfusion of gene transduced cells, patients were treated with a myelosuppressive dose of busulfan (total dose 8mg/kg) intravenously every 12 hours for two days. The busulfan levels in plasma were monitored continuously and cells were infused after 48 hours from the last drug application. L-Bu bioavailability was similar for both patients as revealed by the area under the curve (AUC) of the plasma concentration-time resulting in a median value for the first and third dose of 11,824 ± 229.1 and 12,545 ± 1,126.42 ng·h/ml for P1 and P2, respectively. For P1 the period of myelosuppression (ANC<500 cells/l) lasted 9 days (day 12 to day 21) with a nadir at day 14 (ANC: 60 cells/µl), while myelosuppression lasted only 5 days for P2 (day 13 to day 18) with a nadir at day 15 (ANC: 0 cells/µl). Severe lymphopenia (CD4+ counts <200/µl) was observed in P1 between days +21 and +32, while lymphopenia in P2 was observed only at day +17. Cell counts recovered gradually to the normal values observed prior to busulfan conditioning (P1: 476 CD4+ cells/µl, age 19; P2: 313 CD4+ cells/µl, day -28). Similar observations were made for CD8+ and CD19+ cells. Full platelet recovery (>100x10⁶ cells/µl) was observed at day 157 for P1 and day 35 for P2.

In both adults gene corrected neutrophils were found at levels between 12 – 31% during the first 4 - 5 months after treatment. Thereafter an increase in the number of gp91phox transduced cells was observed reaching up to 40%-60% of the total peripheral blood granulocytes 10 months after transplantation and remained at this level for the next 12-14 months. Importantly, myeloid cell proliferation was re-

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![Fig. (2). The current CGD gene therapy protocol. Hematopoietic stem cells, CD34+ cells, were mobilized into the peripheral blood by G-CSF administration, harvested by leukapheresis and purified under GMP conditions by CliniMACS Column. During ex-vivo GMP transduction of CD34+ cells by retroviral vector, patients were conditioned with Busulfan (8mg/kg total) intravenously. After conditioning, gene-modified cells were infused into the patients.](image-url)
stricted and did not result in granulocyte numbers exceeding those observed before gene therapy [Ott et al., 2006]. Gene marking in CD19+ purified cells was much lower and ranged between 10% and 20%, while gene marking in CD3+ cells was almost negligible. The fact that an increase in gene marked cells was not observed in the lymphoid compartment suggests that mainly the myeloid compartment was affected by the expansion of gene transduced cell. Alternatively, gene transfer may have occurred preferentially into myeloid progenitor cells leading to the expansion of myeloid precursors. To elucidate the molecular basis of the cell expansion, the retroviral integration sites were mapped throughout time by linear-amplification mediated PCR (LAM-PCR) [Schmidt et al., 2002; Schmidt et al., 2003]. This analysis revealed that cell expansion was triggered by activating insertions in the zinc finger transcription factor homologs MDS1/EVI1, PRDM16, or in SETBP1. The increase in gene marking from day 150 on correlates with the appearance of cell clones containing these insertions. This analysis also revealed that the hematopoietic expansion was first polyclonal, then oligoclonal in origin and therefore multiple clones were contributing to final hematopoiesis in both patients, although some of the clones became predominant throughout time and dominated the population of gene marked cells. Similar integration sites were found in CD15+, CD19+ and CD3+ cells providing clear evidence that transduction of multipotent progenitors or even hematopoietic stem cells occurred in both patients.

Gene marking in bone marrow derived colony forming cells (CFC) was also high. For P1 gene marking in CFC was 68.8% at day 122, while 42.8% of the CFC obtained from P2 at day 245 contained proviral sequences. Gene marking was equally distributed among CFU-GM and BFU-E colonies and most of them contained proviral integrations into MDS1/EVI1 or PRDM16.

The expansion of gene marked myeloid cells provided a significant amount of oxidase positive phagocytes and undoubtedly contributed to the success of this clinical trial. Initially the amount of functionally corrected cells was sufficient to eradicate therapy refractory bacterial and fungal infections from which patients had suffered before gene therapy. Thus, this treatment provided a substantial therapeutic benefit to both patients early after transplantation. A closer examination of the amount of superoxide produced by the individual cells revealed that phagocytes derived from P1 had about 1/3 of the superoxide production of normal phagocytes (4.13 vs. 14.35 nmol O₂/10⁶ cells/ min, while cells from P2 produced only 1/8 of normal levels. This correlates with the amount of proviral copies found per cell in P1 (2 copies) and P2 (1 copy) and suggest that even small amounts of superoxide production have therapeutic relevant effects. Indeed isolated granulocytes from P1 were able to ingest and kill bacteria in an in vitro assay clearly proving the functional correction of X-CGD phagocytes.

Despite these observations and the positive results obtained from both adults within the first two years as well as from one 5 year old boy treated similarly in Zurich, one of our patients (P1) died of severe bacterial sepsis after colon perforation 27 months after gene therapy. The cause of death is still under investigation but from the preliminary data available it seems that the amount of functionally corrected cells significantly decreased with time. The second adult patient is free of infection until last time point of monitoring (month 28) and enjoys a normal life. Although the expansion of gene modified cells could suggest the development of an abnormal hematopoiesis, none of the patients have shown signs of myelodysplastic syndrome or even leukemia.

CONCLUSIONS AND FUTURE WORK

Our findings indicate that the genetic modification of human myelopoiesis is feasible and effective. Bacterial and fungal infections that were refractory to conventional treatment could be eliminated after gene therapy. Although the initial numbers of gene corrected cells were substantial and contributed to the eradication of preexisting bacteria and fungal infections, it is difficult to predict whether the levels of transduced granulocytes in vivo would have been maintained over time without the proliferative advantage conferred by retroviral insertion. Nevertheless, abnormal cell expansion is not a desired outcome of any gene therapy trial and modifications in vector design are underway to minimize the effects of insertional mutagenesis. Among others the use of self-inactivating (SIN) vectors which lacks the potent enhancer elements present within the viral LTR has to be considered. Moreover transgene expression in SIN vectors is driven by an internal promoter, which allows for the use of tissue specific promoters thereby further reducing the probability of oncogene activation in stem/progenitor cells. Efficient SIN gammaretroviral vectors have been developed [Kraunus et al., 2004; Schambach et al., 2006] and the first clinical trials with these vectors will soon be performed. Also HIV-1 derived vectors (lentiviral vectors) may offer further improvements in safety since these vectors integrate preferentially within genes as opposed to gammaretroviral vectors, which integrate preferentially in the proximity of promoter regions [Wu et al., 2003]. The use of lentiviral vectors is also associated with a reduced manipulation of HSC ex vivo, since this type of vectors allows gene transfer into quiescent cells and therefore does not require extensive pre-culture of cells with cytokines. Furthermore the inclusion of chromatin insulators may further reduce the possibility of transactivation of genes close to the viral integration site [Bank et al., 2005; Emery et al., 2005]. Lentiviral vectors have been used for the correction of CGD in tissue culture and animal models. Respiratory burst activity was restored in PLB985 X-CGD cells transduced with a SIN lentiviral vector which contained the CMV promoter to drive gp91 transgene expression [Saulnier et al., 2000]. A second study reported correction of NADPH oxidase activity in human CD34+ PBSCs ex vivo as well as in vivo in NOD/SCID mice [Roesler et al., 2002]. Lentiviral vectors have already been used in a clinical trial aimed at the protection of hematopoietic cells from HIV-1 infection [Strayer et al., 2005] and more trials are expected in the future [Bank et al., 2005].

Further considerations may have an impact on the safety application of gene therapy including determination of the minimal vector and cell doses required for the achievement of a therapeutic effect.

Based on our observations we recommend to use LTR-driven retroviral vectors only for those CGD patients in late...
stage of the disease including those suffering from therapy refractory infections or progressive organ dysfunction without further options for HSCT/BMT or no available HLA identical donor. In future the use of modified vectors together with restriction of transgene expression to the myeloid compartment will improve the safety and efficacy of this treatment allowing a wider use of this technology for the benefit of CGD patients in early stage disease and possibly other myeloid related diseases.

An interesting alternative could be to combine gene therapy with allogeneic stem cell transplantation. For example gene therapy could be applied first to eradicate active bacterial and fungal infections followed 1-2 years later by matched stem cell transplantation to provide long-term engraftment and physiological amounts of superoxide production. This sequential procedure could minimize the risks associated with inflammatory flare-up (e.g. Aspergillosis) in immunosuppressed patients undergoing bone marrow transplantation.

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ABBREVIATIONS

CGD = Chronic Granulomatous Disease
NADPH = Nicotinamide Dinucleotide Phosphate Oxidase Complex
ROS = Reactive Oxygen Species
HSCT = Hematopoietic Stem Cell Transplantation
GvHD = Graft versus Host Disease
BMT = Bone Marrow Transplantation
SCID = Severe Combined Immunodeficiency

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