学会発表

Kume A, Matsushita T, Mizukami H, Urabe M, Okada T, Ozawa K:	Long-term efficacy of a self-complementary adeno-associated virus vector for phenylketonuria gene therapy	The 10th Annual Meeting of the American Society of Gene Therapy	2007.May30-June3	Seattle, WA, USA.	2007.
Onodera M;	Gene Therapy for Hematologic Malignancy.	American Society of Gene Therapy. The 10th Annual Meeting of the American Society of Gene Therapy,	2007.May30-June3	1) Seattle, WA, USA.	2007
Ariga T:	International Symposium: Gene Therapy Clinical Trials from Around the Globe, Hematopoietic stem cell gene therapy for two patients with Adenosine Deaminase (ADA) deficiency without myelopreparative conditioning; a suggestion for the optimal protocol for HSC gene therapy for ADA deficiency.	The 10th Annual Meeting of the American Society of Gene Therapy,	2007.May30-June3	2) Seattle, WA, USA.	2007
Onodera M	Opposite effects of an integration site on expression of the transgene-Genesilencing or Leukemogenesis	IVth Conference on Stem Cell Gene Thrapy.	2007.Sep13-17	Thessalonikki, Halkidiki, Greece.	2007

Ariga T:	Hematopoietic stem cell (HCS) gene therapy for two patients with adenosine deaminase (ADA) deficiency without cytoreductive conditioning; a suggestion for the optimal protocol for HSC gene therapy for ADA deficiency.	Symposium VI Immune deficiency syndromes. The IVth Conference on Stem Cell Gene Therapy.	2007.Sep13-17,	Halkidiki, Thessalonikii, Greece,	2007
Otsu M, et al.	A novel hematopoietic stem cell transplantation strategy utilizing pre-infusion of donor lymphocytes.	The 15th Annual Congress of the European Society of Gene and Cell Therapy,	2007.27-30 October	Rotterdam, the Netherlands,	2007
Kume A, Matsushita T, Mizukami H, Urabe M, Okada T, Ozawa K:	Efficient and stable liver transduction by a self-complementary adeno- associated virus vector for phenylketonuria gene therapy	第13回日本遺伝子治療学会	2007.6.28-6.30	3) 名古屋	2007
Horiuchi Y, Otsu M, Kiyokawa N, Migita O, Ogata T, Li XK, Onodera M, Kume A, Okuyama T, Fujimoto J, Nakauchi H, Kuratsuji T;	Towards clinical gene therapy for chronic granulomatous disease: Optomization of gene transduction into hematopoietic stem/progenitor cells.	第13 回日本遺伝子治療学会	2007.6.28-6.30	名古屋	2007
Nabekura T, Nagasawa T, Nakauchi H, Onodera M	An immunotherapy approach with dendritic cells genetically modified to express the tumor-associated antigen, HER2.	日本免疫学会	2007.11.20～2007.11.22.	東京	2007
久米晃啓、松下卓, 小倉剛,小澤敬也:	自己相補型アデノ随伴ウイルスベクターによるフェニルケトン尿症遺伝子治療	第110回日本小児科学会小児科学会学術集会	2007年4月	京都.	2007
大津 真, 他.	アロT細胞先行投与による新規造血幹細胞移植法	第69回日本血液学会,	2007年10月	横浜	2007

田口智子, 宮川世志幸, 堀内保臣, 斎藤洋平, 竹野内寿美, 北村紀子, 松井淳, 佐藤伴, 鈴木恭子, 斎藤正博, 片桐洋子, 大喜多肇, 藤本純一郎, 清河信敬	ncostatin Mの造血調節作用に関するin vitroでの検討	第69回日本血液学会総会・第49回日本臨床血液学会総会合同開催	10月11日-13日, 2007	横浜	2007
大津 真.	ADA 欠損症における日本独自の遺伝子治療	第49回日本小児血液学会,	2007 年 12 月	仙台,	2007
堀内保臣, 清河信敬, 宮川世志幸, 中島英規, 斉藤洋平, 右田王介, 片桐洋子, 大喜多肇, 岡田真由美, 大津真, 小野寺雅史, 久米晃啓, 犬飼岳史, 奥山虎之, 杉田完爾, 藤本純一郎, 倉辻忠俊	ヒト造血幹細胞に対する組換えフィブロネクチンを用いたレトロウイルス感染の分子機構に関する検討	第49回日本小児血液学会総会・第23回日本小児がん学会学術集会 合同開催	2月14日-16日, 2007.	仙台	2007
岡田真由美, 奥山虎之, 立澤宰, 小林信一, 清河信敬, 大津真, 藤本純一郎, 倉辻忠俊,	X連鎖慢性肉芽腫症(X-CGD)に対する遺伝子治療臨床研究,	第15回食細胞機能異常症研究会	2007年12月14日	東京	2007
岡田真由美, 奥山虎之, 立澤宰, 小林信一, 清河信敬, 藤本純一郎, 倉辻忠俊, 大津真, 有賀正, 久米晃啓, 小野寺雅史, 布井博幸,	世界の慢性肉芽腫症遺伝子治療臨床研究と日本の現状,	第1回日本免疫不全症研究会,	2008 年 1 月 19 日	東京	2007

別添

研究成果の刊行物・別冊

Chapter 1

Stem Cell Gene Therapy for ADA-Deficiency without Myelopreparative Conditioning

Makoto Otsu*,† and Tadashi Ariga*,‡

Severe combined immunodeficiency (SCID) due to adenosine deaminase (ADA)-deficiency has been one of the best target diseases for clinical gene therapy. Stem cell gene therapy (SCGT) mediated by *ex vivo* transduction of hematopoietic stem cells with an *ADA* transgene can be curative for ADA-SCID patients, having a potential to enable life-long immune reconstitution. We have treated two ADA-SCID patients by retroviral-mediated gene transfer into bone marrow $CD34^+$ cells. Of note, we did not give any myelopreparative conditioning to our patients before cell infusion, while the concurrent successful SCGT trials utilized chemotherapy with the aim of enhancing engraftment of infused gene-corrected cells. At ~3 years post SCGT, clinical benefits are evident for both patients even in the absence of myelopreparative conditioning. Gene-corrected $CD34^+$ cells have engrafted in both patients and still keep producing mature hematopoietic cells expressing ADA levels sufficient to allow systemic detoxification. Our SCGT trial has also been successful in providing protective immunity to our two patients. The extent and kinetics of immune reconstitution, however, are significantly different from those achieved in the SCGT trials with preconditioning. These results indicate that preconditioning-free SCGT for ADA-deficiency may be further improved since it does not yet match the clinical outcomes of concurrent preconditioning-based SCGT trials. Unlike the pioneering SCGT trial for X-SCID in which success has been plagued by some random-integration oncogenic cases, all ADA-SCID SCGT trials, including ours, are thus far free from adverse events. However, myelopreparative conditioning might have a negative effect on the long-term repopulating potential of the HSC pool. Therefore, our protocol through worldwide cooperation will eventually culminate in the

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establishment of a long-term risk-free SCGT protocol for ADA-deficiency, thereby maximizing the effectiveness of current successful myelopreparative-conditioning SCGT strategies.

Keywords: Adenosine deaminase; severe combine immunodeficiency; gene therapy; clinical trials; hematopoietic stem cells; myelopreparative conditioning.

1. Introduction

Severe combined immunodeficiency, SCID, is a heterogeneous group of disorders with which affected patients have profound defects in their immune functions (Buckley, 2004; Fischer *et al.*, 2005). Among the currently recognized SCID diseases, adenosine deaminase(ADA)-deficiency represents ~15% of all cases (Buckley *et al.*, 1997; Stephan *et al.*, 1993). ADA-deficiency has been one of the best target diseases for clinical gene therapy since the pioneering trial conducted in 1990, which was originally aimed at genetic correction of patients' T cells (Blaese *et al.*, 1995). Extensive efforts have been made to improve efficacy and safety of genetic correction for ADA-deficiency either by targeting patients' T cells or hematopoietic stem cells (HSCs) (Onodera *et al.*, 1998a; Hoogerbrugge *et al.*, 1996; Kohn *et al.*, 1995; Bordignon *et al.*, 1995). These efforts have culminated in the recent success achieved in the stem cell gene therapy trial conducted by Aiuti *et al.*, which has for the first time introduced nonmyeloablative conditioning for stem cell gene therapy (SCGT) (Aiuti *et al.*, 2002a). In this trial, the use of a low-dose of busulfan as a cytoreductive reagent is believed to have played a key role for the beneficial immune reconstitution achieved in patients by helping efficient engraftment and expansion of gene-corrected HSCs. Another successful trial reported by Gasper *et al.* that used melphalan as a preconditioning drug has further supported the idea that creating some "space" in patient's marrow can lead to a better outcome in SCGT for ADA-deficiency (Gaspar *et al.*, 2006). Although possibly in rare instances, however, there seem to be some cases in which the use of chemotherapeutic reagents may not be appropriate because of unpredictable hypersensitivity of patients' hematopoietic cells to the drugs (Engel *et al.*, 2007). Moreover, since the long-term outcomes of treated patients still remain to be determined, it may be desirable to keep seeking for the ultimate treatment procedures that can provide ADA-deficient SCID patients with life-long immune reconstitution while simultaneously eliminating any treatment-related risks.

Of note, we have had our own clinical trial of SCGT in which two ADA-deficient SCID patients received autologous gene-corrected HSCs without any myelopreparative conditioning. Both patients clearly obtained clinical

benefits and have no need for ADA enzyme replacement for more than 3 years with partial, but still protective reconstitution of their immunity. In this chapter, we will summarize our clinical trial and try to make a comparison between different concurrent trials. We believe that the results of our trial can add important pieces of information to the field of SCGT and that the collective efforts will eventually lead to the establishment of an ideal treatment for ADA-deficiency.

2. Severe Combined Immunodeficiency due to ADA-deficiency

ADA is the purine salvage enzyme ubiquitously expressed in mammalian cells. Genetic defects in the *ADA* gene that is located on chromosome 20 lead to accumulation of cellular toxic metabolites, which can cause death of lymphocytes and consequently profound deficiency in immune systems (Markert, 1994; Hirschhorn, 1993; Giblett *et al.*, 1972). Systemic involvement due to metabolic toxicities of various non-lymphoid organs, including liver, kidney and gut is also a feature in ADA-deficient SCID patients, making this disorder distinctive from other major SCID diseases such as X-linked SCID. Because ADA-deficiency is virtually a "metabolic" disease, the curative treatment requires correction of not only immune deficiency, but also generalized toxicities.

Allogeneic bone marrow transplantation (BMT) or HSC transplantation (HSCT) has been successful as a curative treatment for ADA-deficiency when HLA-identical sibling donors are available (Myers *et al.*, 2002). Because the lack of such ideal donors is not uncommon for most patients, BMT from HLA-haploidentical parental donors have been performed with limited success (Haddad *et al.*, 1998; Buckley *et al.*, 1999). HLA-matched unrelated donor procedures are also shown to result in frequent transplantation failure, making ADA-deficiency one of hard-to-manage SCID phenotypes (Booth *et al.*, 2007).

The presence of another life-saving option is one remarkable feature of ADA-deficiency; polyethylene glycol-modified ADA (PEG-ADA) was developed as an enzyme replacement reagent (Hershfield, 1995). PEG-ADA has provided over 150 patients worldwide with life-saving effects, but recent reports have highlighted still protective, but somehow limited immune reconstitution observed in treated patients with long follow-up (Chan *et al.*, 2005; Malacarne *et al.*, 2005). Despite the above-mentioned limitation, it is still true that PEG-ADA stands as one of the major life-saving treatment options for ADA-SCID capable of stabilizing patients prior to other curative treatments such as HSCT and SCGT (Booth *et al.*, 2007).

3. Gene Therapy Trials for ADA-deficiency

3.1. *T Cell-Directed Gene Transfer as a Treatment for ADA-deficiency*

In the early clinical trials including ours (Onodera *et al.*, 1998a), peripheral T lymphocytes were used as target cells for gene transduction. These trials have demonstrated that retroviral-mediated gene transfer could be safely completed and lead to long-term gene marking/expression in patients' T cells (Blaese *et al.*, 1995; Bordignon *et al.*, 1995; Onodera *et al.*, 1998a). Although limited ADA-transgene expression in the T cell compartment seems sufficient to provide protective immunity with the concomitant use of PEG-ADA, it likely fails to supply ADA in sufficient amounts to detoxify systemic organs (Aiuti *et al.*, 2002b).

3.2. *Early Stem Cell Gene Therapy Trials*

To achieve life-long immune reconstitution by gene therapy, targeting HSCs is thought to be the ideal measure because of their ability to self-renew and differentiate into multiple hematopoietic lineages. Several pioneering trials aimed at genetic correction of HSCs via retroviral-mediated gene transfer have been conducted (Bordignon *et al.*, 1995; Kohn *et al.*, 1995; Hoogerbrugge *et al.*, 1996). These trials resulted in long-term engraftment of gene-marked cells, thereby establishing that hematopoietic progenitor/stem cells with repopulating abilities can be targeted by retroviral-mediated gene transfer (Schmidt *et al.*, 2003). Treated patients, however, did not gain clear clinical benefits from transduced cell populations. The reasons for poor immune recovery in these trials are now thought to be largely attributable to insufficient gene transduction into HSCs and to impairment due to continuous use of PEG-ADA of selective growth/survival advantages normally expected for gene-corrected cells.

3.3. *Second Generation of Stem Cell Gene Therapy Trials for ADA-deficiency*

Based on the cumulative experience of early clinical trials and the advancement in gene transfer techniques into HSCs, a team in Italy started the new generation of SCGT trials for ADA-deficiency (Aiuti *et al.*, 2002a). In sharp contrast to the early trials, the treatment provided most patients with systemic detoxification and good immune reconstitution, thereby eliminating the need for PEG-ADA administration. This trial has introduced two major

amendments in its protocol, i.e., to treat patients who are not on PEG-ADA and to use a low-dose of busulfan as a preconditioning reagent. These two modifications have most likely played important roles in the improvement of the clinical outcomes. Usefulness of mild preconditioning for SCGT has been further confirmed by a report from Gaspar *et al.*, which demonstrated meaningful immune reconstitution in an ADA-SCID patient treated following the Italian SCGT protocol but using Melphalan instead of busulfan (Gaspar *et al.*, 2006). Together with two other clinical trials, one conducted in the USA (Engel *et al.*, 2007) and the other by us in Japan (see below), SCGT is now establishing its status as a curative treatment option for ADA-deficiency. We believe that cumulative experience from different trials will help establish the best treatment protocol for SCGT for ADA-deficiency.

4. Stem Cell Gene Therapy for ADA-deficiency without Myelopreparative Conditioning

In 1995, we started the clinical trial that was aimed at full genetic correction of patient T cells by retroviral-mediated gene transfer. Although the patient clearly benefited from the treatment, we could not withdraw PEG-ADA replacement because gene-marked T cells alone seemed incapable of producing sufficient ADA for systemic detoxification (Kawamura *et al.*, 1998; Onodera *et al.*, 1998a; Kawamura *et al.*, 1999). While seeking for a better treatment option for this patient, we happened to have another ADA-deficient SCID patient who did not have a HLA-identical sibling donor (Ariga *et al.*, 2001). PEG-ADA had helped her growth and raised her immune functions to a level compatible with a transition from hospital to day care, but she had to stay home most of the time in order to avoid severe infections. We therefore decided to conduct the clinical trial of stem cell-directed gene therapy with the aim of giving patients sustained immune reconstitution by eliminating the necessity for supportive treatment including PEG-ADA.

The above-mentioned two ADA-deficient SCID patients (pt 1, a 4-year-old girl and pt 2, a 13-year-old boy) have been enrolled in the trial. PEG-ADA was stopped ~5 weeks prior to treatment. Bone marrow (BM) cells were collected and then subjected to $CD34^+$ cell-purification procedures. We then transduced $CD34^+$ cells ($\sim 1 \times 10^6$ cells/kg) with the retroviral vector GCsapM-ADA (Onodera *et al.*, 1998b) pseudotyped with gibbon ape leukemia virus (GALV) envelope. We used for cell culture, a serum-free medium supplemented with a combination of cytokines: SCF, TPO, Flt3-L, IL-6, and soluble IL-6R (sIL-6R). After 3 rounds of transduction, we obtained $\sim 1 \times 10^6$ cells/kg of $CD34^+$ cells that expressed high levels of ADA

with ~50% of cells transduced. Gene-corrected autologous BM cells were intravenously injected into pt 1 on December 2003 and into pt 2 on February 2004 without any preconditioning. No acute adverse reaction was noted.

For the first several weeks in the absence of PEG-ADA replacement, some symptoms related to ADA-deficiency, including mild anorexia became evident in both patients. Consistent with the clinical ADA-deficient state, the levels of accumulated toxic metabolites measured in erythrocytes reached the levels of the untreated ADA-deficient SCID patients. Liver toxicity was also noted (Fig. 1). Starting from as early as 1 week post SCGT, however, these ADA-deficiency-related abnormalities gradually disappeared. Of note, liver enzyme values completely normalized for both patients (Fig. 1), and toxic metabolite values continuously remain low for up to ~3 years post treatment, the levels being similar to those achievable in successful HSCT cases. These results indicate that SCGT without myelopreparative preconditioning can lead to systemic detoxification in ADA-deficient SCID patients.

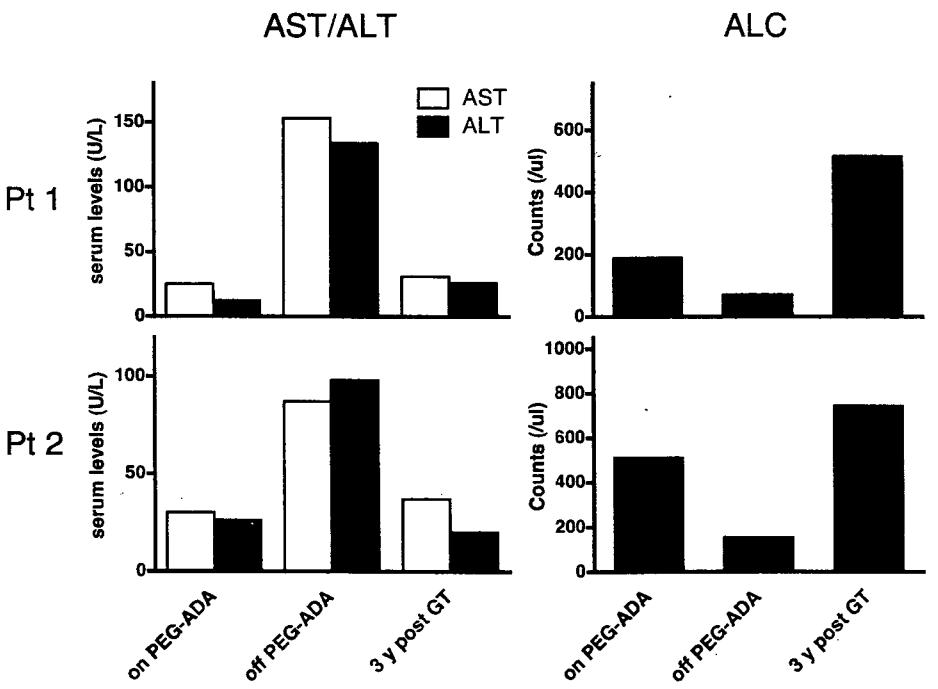

Fig. 1. Effectiveness of stem cell gene therapy for ADA-deficiency without myelopreparative preconditioning. *Left:* Serum levels of liver enzymes. Serum aspartate aminotransferase (AST, empty columns) and alanine aminotranferase (ALT, solid columns) activities are shown before cessation of PEG-ADA (on PEG-ADA), at peak levels (38 and 41 days after cessation of PEG-ADA for pt 1 and pt 2, respectively), and ~3 years after gene therapy (3 y post GT). *Right:* Absolute lymphocyte counts (ALC). Time points are the same as for serum levels of liver enzyme activities.

Reconstitution of hematopoiesis and immune functions was also achieved, although with much slower kinetics than those observed in other trials that used mild preconditioning (Aiuti *et al.*, 2002a; Gaspar *et al.*, 2006). Lymphocyte counts showed a clear increase from ~4–6 months post treatment, then reached levels higher than those observed in their pre-SCGT status (300–400/ μl for pt 1 and 500–700/ μl for pt 2; Fig. 1). Immunophenotyping of these lymphocytes revealed that T cells represented a major subset of polyclonal nature as evidenced by TCR-spectratyping analysis. Accordingly, proliferative responses of T cells to mitogenic stimuli were remarkably improved in both patients. In contrast, increase of B and NK cells did not occur until ~10 months after treatment for pt 1. Once developed, however, these lymphocytes showed steady improvement of their counts. This delayed reconstitution is in sharp contrast to that achieved in the Italian trial that showed quick recovery of B and NK cells starting as early as 2 months post treatment (Aiuti *et al.*, 2002a). For pt 2, NK cell counts increased, although to a lesser extent, while B cell counts remained low throughout the observation period. Because antibody production after SCGT is currently considered insufficient, both patients are still on immunoglobulin replacement therapy. Overall, SCGT has improved both patients' immunity to levels compatible with protection from life-threatening infections without the need for PEG-ADA administration.

Assessment of gene-marking levels revealed that virtually ~100% of T cells possessed the transgene in both patients. Similar analysis was available for B and NK cells only for pt 1, and showed ~60% and ~80% marking levels for B cells and NK cells, respectively. It is noteworthy that we could confirm the presence of transgene in ~10% of colony-forming cells derived from BM at ~3 years post SCGT. Similar marking levels determined in BM $CD34^+$ cells by quantitative PCR assay further support the idea that significant portions of hematopoietic stem/progenitor cells have been successfully transduced and stably engrafted in patients' marrow without cytoreductive preconditioning.

We have carried out sequential analysis of retroviral integration sites in peripheral blood mononuclear cell samples, and have demonstrated the oligoclonal nature of transduced cells. During the ~3 year-observation period, we have not observed in either patients any severe adverse events, including leukemic expansion of abnormal clones. At the time of writing, pt 1 is an elementary school pupil while pt 2 is a high school student. Although we have not achieved complete withdrawal of supportive treatment, e.g., prophylactic antibiotics, it may be of great significance that both patients have been off PEG-ADA for as long as three years without serious infectious episodes. Further follow-up is necessary to evaluate the long-term

safety and efficacy of SCGT for ADA-deficiency without myelopreparative conditioning.

5. Factors that Likely Affect the Safety and Efficacy of Stem Cell Gene Therapy for ADA-deficiency

To establish the ultimate protocol with the maximal efficacy and minimal risk, it is valuable to consider what factors likely affect patient outcomes after SCGT. Although there should be more than those listed below, we will focus on the five factors that are considered in particular to have influenced the outcome of our patients.

5.1. *Age of Patients*

Our patients were relatively old (4-year-old and 13-year-old) at the beginning of SCGT. From the clinical experience obtained from either BMT (Myers *et al.*, 2002; Buckley *et al.*, 1999) or SCGT (Hacein-Bey-Abina *et al.*, 2002; Cavazzana-Calvo *et al.*, 2000) for SCID diseases, younger age of patients seems critical for treatment effectiveness. This is probably due to several reasons that include higher quality of "younger" HSCs and better patients' condition with less frequent histories of infections. Of note, SCGT trials for another SCID disease, i.e., X-linked severe combined immunodeficiency (X-SCID) indicated a possible age-related limitation for treatment efficacy (Thrasher *et al.*, 2005). Although particularly young age (less than 6 month-old) may be correlated to the high incidence of leukemia-like adverse events reported in the French X-SCID gene therapy trial (Hacein-Bey-Abina *et al.*, 2003a), younger age should be considered as one of the critical factors for successful SCGT.

5.2. *Transduction Procedures*

Currently, all the clinical trials of SCGT for SCID have utilized retroviral vectors for gene transfer. Because cell division is a prerequisite for retroviral-mediated gene transduction, various combinations of cytokines are being used in each trial in order to stimulate BM $CD34^+$ cell proliferation. Ideal transduction procedures, we believe, should preserve the life-long repopulating/multi-differentiating capabilities of transduced stem cells without impairing good transduction efficiency. Our protocol is the sole one that utilizes IL-6 and sIL-6R besides the standard cytokines, SCF, TPO, and Flt-3L. Our protocol may thus be significantly different from all others.

Addition of IL-6/sIL-6R was expected to enhance repopulating abilities of human $CD34^+$ cells based on experiments utilizing a NOD/SCID xenotransplantaion model (Ueda *et al.*, 2000) and therefore might have contributed to the favorable clinical outcomes of our patients. As a newer xenotransplantation model, NOD/SCID/IL-2Rγ chain KO mice, has recently become available with its improved capability to allow human T cell development (Shultz *et al.*, 2005; Ishikawa *et al.*, 2005). Further refinements of cytokine combinations may thus be experimented that might be instrumental for the future clinical trials.

5.3. *Cell Doses*

Accumulation of worldwide clinical experience has generally demonstrated a positive correlation between higher cell doses and better engraftment (Davies *et al.*, 2000; Bittencourt *et al.*, 2002). A large number of murine experiments have also supported this correlation (Rao *et al.*, 1997; Mauch and Hellman, 1989; Glass *et al.*, 1993). The relatively low doses of $CD34^+$ cells ($\sim 1 \times 10^6$ cells/kg) transplanted into our patients may thus explain, at least partially, the much slower hematopoietic recovery than those observed in the other successful SCGT cases (Hacein-Bey-Abina *et al.*, 2002; Aiuti *et al.*, 2002a). In the SCGT settings, however, the cell dose effect on clinical outcome should be carefully discussed. During a 4–5 day course of gene transduction, $CD34^+$ hematopoietic cells usually expand to certain extents, which will vary according to the difference in cytokine cocktails. Extreme expansion of $CD34^+$ cells, however, does not necessarily lead to HSC expansion but rather may compromise the quality and/or quantity of HSCs. It is therefore important to consider the balance between maintenance of good repopulating abilities and total cell expansion when evaluating the appropriateness of culture conditions. Since single injections of relatively small numbers of gene-corrected cells have led to the development of hematopoietic cells capable of maintaining ADA levels sufficient for systemic detoxification, we may assume high repopulating abilities in the infused $CD34^+$ cell populations. Obviously, higher numbers of transduced cells with a similar repopulation potential would enhance hematopoietic and immunological reconstitution with our SCGT protocol.

5.4. *Use of PEG-ADA*

PEG-ADA has been, and will remain indispensable as a treatment option for ADA-deficiency. It especially has great significance in that the drug can

be used to promptly stabilize patients' condition when HLA-matched suitable donors turn out to be lacking (Booth *et al.*, 2007). PEG-ADA, however, does have a disadvantage in terms of "selective growth/survival advantage for gene-corrected cells," which must be taken into account in the settings of SCGT. Since PEG-ADA helps growth/survival of both uncorrected and gene-corrected patient cells, concomitant use of the enzyme replacement is believed to lower the treatment efficacy by impairing the advantage otherwise assigned to ADA-normalized cells. This idea is supported by the expansion of small numbers of spontaneously gene-corrected T cells in the reported case of *in vivo* gene-reversion (Hirschhorn *et al.*, 1996) and the results of earlier clinical gene therapy trials for ADA-deficiency (Aiuti *et al.*, 2002b; Kohn *et al.*, 1998). The idea that we could expect better selective expansion of gene-corrected cells if PEG-ADA is not given to patients at the time of SCGT, does now translate into PEG-ADA-free SCGT trials for ADA-deficiency.

5.5. *Cytoreductive Preconditioning*

Apparently, the most remarkable difference between our protocol and the other currently ongoing SCGT clinical studies is in the use of cytoreductive preconditioning. The trials running in Italy, the UK, and the US are all utilizing a chemotherapeutic reagent before infusion of gene-transduced BM cells (Gaspar *et al.*, 2006; Engel *et al.*, 2007; Aiuti *et al.*, 2002a), while we did not give any preconditioning treatment to both patients enrolled in our trial. Although the above-mentioned factors, including age and cell doses may also have affected our patients' outcome, it may be natural to consider that the absence of preconditioning has significantly influenced the kinetics of hematopoietic reconstitution. Conditioning regimen, including "no preconditioning" as well, is a matter of debate in the SCGT field for primary immunodeficiency diseases that include not only ADA-deficiency but also other disorders. The conditioning issue will greatly affect the evaluation of the risk/benefit balance in each SCGT trial. We will, therefore, pay particular attention to this issue and discuss the significance of our own trial in light of the current situation in the SCGT field in the following section.

6. Myelopreparative Treatment: Roles in Stem Cell Gene Therapy

6.1. *Concept of Myelopreparation*

It has been a widely accepted concept that the use of cytoreductive treatment as conditioning makes "space" in recipient BM to give transfused HSCs a

better chance to engraft (Vriesendorp, 2003). It is also true, however, that engraftment of long-term repopulating HSCs does not necessarily require marrow-conditioning as shown in many murine experimental studies (Stewart *et al.*, 1993; Bhattacharya *et al.*, 2006). Quesenberry *et al.* have shown that the replacement of HSCs likely occurs according to "equilibration" between host- and donor-cell populations in a setting of syngeneic BMT (Colvin *et al.*, 2004). We thus may think that the main effect of conditioning, i.e., total body irradiation or chemotherapeutic drugs, is not "to make space" but "to reduce competitive counterparts" (Fig. 2). According to this theory, the use of conditioning in SCGT may have particular significance. As shown in Fig. 2, we may regard SCGT after conditioning as "reductive"

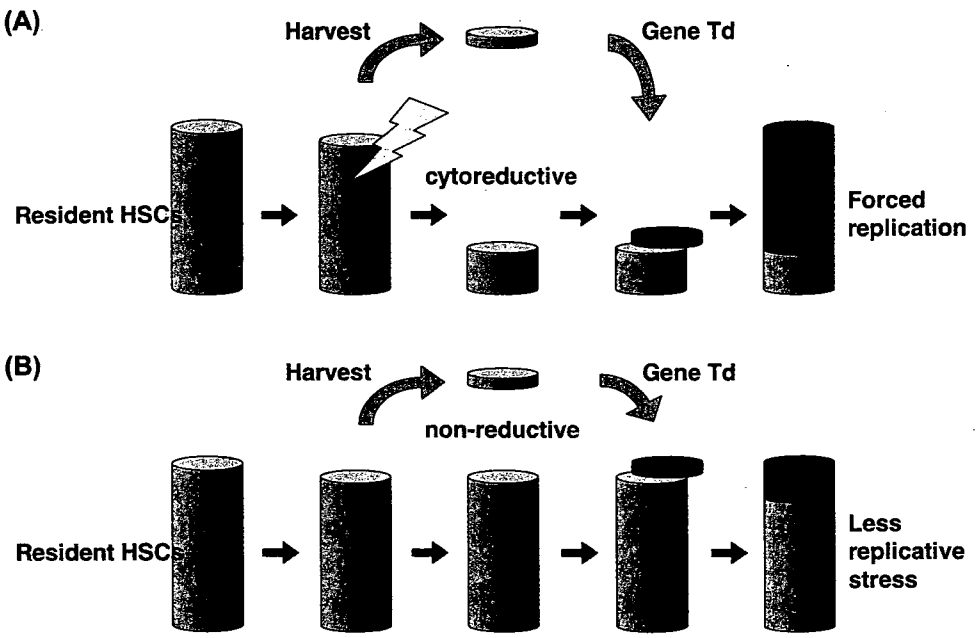

Fig. 2. Schematic representation for modeling of replicative stresses forced on HSCs in stem cell gene therapy. **(A)** Treatment with cytoreductive conditioning. The blue column on the left represents a total pool of patient HSCs. A small portion of the pool (a thin slice of blue column at the top) is removed from patient BM (Harvest), and then transduced with the therapeutic vectors (Gene Td). Gene-corrected HSCs (a thin slice of red column) is given back to BM. Because of cytoreductive conditioning, total mass of the original HSC pool is reduced (smaller blue columns). As a consequence, gene-corrected HSCs are most likely forced to rapidly replicate, resulting in substantial loss of their repopulation abilities. **(B)** Treatment with no conditioning. In the absence of conditioning, the resident HSC pool remains relatively untouched (blue columns). Transfused gene-corrected HSCs (a thin slice of red column) now have to compete for replication with resident HSCs, but are thought to be less susceptible to a replication-related loss of repopulation abilities.

and SCGT with no conditioning as "non-reductive" with regard to the total number of HSCs. In light of the recent advances in stem cell research, senescence of HSCs has become an important issue (Wang *et al.*, 2006; Allsopp *et al.*, 2003). As replicative stress is thought to be one of the major factors that promote HSC senescence in transplantation (Harrison *et al.*, 1990), non-reductive SCGT may have an advantage in terms of the preservation of long-term repopulation potential in HSCs.

6.2. *Conditioning in SCGT: comparison between ADA-SCID and X-SCID*

Primary immunodeficiency represents the exceptional example regarding the choice of conditioning in transplantation medicine, especially for the cases of SCID. First, in the settings of allogeneic HSCT, preconditioning as a measure of immune suppression can be precluded since the patients' immunity is already impaired. Second, as SCGT utilizes patient's autologous hematopoietic cells, it also precludes the need for immunosuppressive drugs unless the strong immune reaction against the transgene product becomes a issue. Finally, selected SCID diseases such as X-SCID may not necessitate a high chimerism of donor- or gene-corrected-cells from the use of cytoreductive conditioning because of creditable selective growth/survival advantages assigned to normal or normalized cells over diseased cells. In fact, it is believed that such advantages have played a major role in achieving successful reconstitution in patients' immune in the X-SCID gene therapy trials (Cavazzana-Calvo *et al.*, 2000; Gaspar *et al.*, 2004). We may expect similar selective advantage in SCGT for ADA-deficiency, as well, considering the reported ADA-SCID cases in which spontaneously gene-corrected (revertant) T cells showed preferential expansion in the context of ADA-deficient counterparts (Ariga *et al.*, 2001; Hirschhorn *et al.*, 1996). Recent studies that used ADA-deficient mouse models also support this idea (Mortellaro *et al.*, 2006). Nonetheless, T cell reconstitution after transplantation without conditioning seems less satisfactory in the cases of ADA-deficiency than those of X-SCID in both HSCT (Haddad *et al.*, 1998; Buckley *et al.*, 1999) and SCGT settings. As suggested in a recent report, this advantage for X-SCID may be attributed to the absence of resident T cell precursors and the intact thymic environment (Cavazzana-Calvo *et al.*, 2007). Since the developmental block of T cells is not a feature of ADA-deficiency while it is the case for X-SCID, resident T cell precursors likely exist in ADA-deficient thymic environment with the potential to compete with incoming "normal" precursors. In addition, the thymic environment itself may be somehow dysfunctional

in the absence of ADA, thereby hampering the prompt repopulation of incoming T cell precursors. Finally, since ADA-transduced transplanted cells have a systemic therapeutic action (toxic metabolite clearing) in addition to their specific HSC repopulating activity (see above), slow initial kinetics of immune reconstitution might reflect a bi-phasic process in which early systemic detoxification interferes with full immune recovery. By contrast, for X-SCID, the selective advantage of gene-corrected cells should theoretically remain constant throughout the treatment period after SCGT. For B cell development, however, the selective advantage situation appears to be very different between ADA-deficiency and X-SCID. In general, the B cell compartment of X-SCID patients rarely shows high chimerism of donor-derived or gene-corrected B cells without conditioning in both HSCT and SCGT situations (Cavazzana-Calvo *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2002; Buckley *et al.*, 1999; Haddad *et al.*, 1998). This is most likely due to constraints of "normal" B cell development in BM because of competition with resident X-SCID B cell precursors as suggested in murine experiments (Liu *et al.*, 2006; Otsu *et al.*, 2000). In contrast, one patient in our trial has shown steady increase of B lymphocytes, ~60% of which were estimated to be gene-corrected. This observation supports the idea that due to sufficient selective advantage for gene-corrected cells, a limited number of normalized HSCs still have the potential to repopulate B cell compartments to meaningful levels in ADA-deficient SCID patients even in the absence of conditioning.

7. Future of SCGT for ADA-deficiency

Currently, for patients with ADA-deficiency who do not have a HLA-identical sibling donor, SCGT may be regarded as the first treatment option to be tested either before or after stabilization with the use of PEG-ADA. As has been reported above, over 10 ADA-deficient SCID patients have been treated worldwide with the latest SCGT protocols since the first successful cases in Italy, but there have been no treatment-related severe adverse events reported so far. Insertional leukemogenesis has been recognized as an inherited risk of retroviral-mediated gene transfer as evidenced in the French X-SCID gene therapy trial (Hacein-Bey-Abina *et al.*, 2003b and 2003a). In comparison to the cases of X-SCID gene therapy, gene-corrected HSCs expressing the ADA transgene may be less prone to leukemogenic events that are believed to occur due to cooperation between vector-insertional effects and the transgene expression (Thrasher *et al.*, 2006). Careful follow-ups of treated patients are absolutely essential for further development of ADA-SCGT.

Establishment of the ideal treatment protocol for ADA-deficiency is obviously the ultimate goal. The recently developed gene-editing technique using a set of zinc finger nucleases (Urnov *et al.*, 2005) may be utilized for SCGT with the expectation that it can eliminate the risk of insertional leukemogenesis. Even if this becomes close to reality, however, the choice of conditioning will still remain the issue of debate. As long as *ex vivo* manipulation is inevitable in the currently available SCGT protocols, balancing selective advantages given to gene-corrected cells against competition between infused- and resident-HSCs continues to be critical to maximize treatment effectiveness. With the advancement in deciphering HSC-homing/lodgment mechanisms (Quesenberry *et al.*, 2005; Lapidot *et al.*, 2005; Broxmeyer, 2005) and with the improvement in technologies of HSC manipulation (Hofmeister *et al.*, 2007; Zheng *et al.*, 2003), we may expect further enhancement of treatment efficacy of ADA-SCGT even in the absence of conditioning. We believe that worldwide cooperations will culminate in the development of a risk-free SCGT protocol that can grant prompt, sufficient, and life-long immune reconstitution to all the ADA-deficient SCID patients in the not too far future.

References

Aiuti A, Slavin S, Aker M, *et al.* (2002a) Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science* **296**: 2410–13.

Aiuti A, Vai S, Mortellaro A, *et al.* (2002b) Immune reconstitution in ADA-SCID after PBL gene therapy and discontinuation of enzyme replacement. *Nat Med* **8**: 423–25.

Allsopp RC, Morin GB, DePinho R, *et al.* (2003) Telomerase is required to slow telomere shortening and extend replicative lifespan of HSCs during serial transplantation. *Blood* **102**: 517–20.

Ariga T, Oda N, Yamaguchi K, *et al.* (2001) T-cell lines from 2 patients with adenosine deaminase (ADA) deficiency showed the restoration of ADA activity resulted from the reversion of an inherited mutation. *Blood* **97**: 2896–99.

Bhattacharya D, Rossi DJ, Bryder D, Weissman IL. (2006) Purified hematopoietic stem cell engraftment of rare niches corrects severe lymphoid deficiencies without host conditioning. *J Exp Med* **203**: 73–85.

Bittencourt H, Rocha V, Chevret S, *et al.* (2002) Association of CD34 cell dose with hematopoietic recovery, infections, and other outcomes after HLA-identical sibling bone marrow transplantation. *Blood* **99**: 2726–33.

Blaese RM, Culver KW, Miller AD, *et al.* (1995) T lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years. *Science* **270**: 475–80.

Booth C, Hershfield M, Notarangelo L, *et al.* (2007) Management options for adenosine deaminase deficiency, *Proc EBMT Satellite Workshop*, Hamburg, March 2006. *Clin Immunol* **123**: 139–47.

Bordignon C, Notarangelo LD, Nobili N, *et al.* (1995) Gene therapy in peripheral blood lymphocytes and bone marrow for ADA- immunodeficient patients. *Science* **270**: 470–75.

Broxmeyer HE. (2005) Biology of cord blood cells and future prospects for enhanced clinical benefit. *Cytotherapy* **7**: 209–18.

Buckley RH. (2004) Molecular defects in human severe combined immunodeficiency and approaches to immune reconstitution. *Annu Rev Immunol* **22**: 625–55.

Buckley RH, Schiff RI, Schiff SE, *et al.* (1997) Human severe combined immunodeficiency: genetic, phenotypic, and functional diversity in one hundred eight infants. *J Pediatr* **130**: 378–87.

Buckley RH, Schiff SE, Schiff RI, *et al.* (1999) Hematopoietic stem-cell transplantation for the treatment of severe combined immunodeficiency. *N Engl J Med* **340**: 508–16.

Cavazzana-Calvo M, Carlier F, Le Deist F, *et al.* (2007) Long-term T-cell reconstitution after hematopoietic stem-cell transplantation in primary T-cell-immunodeficient patients is associated with myeloid chimerism and possibly the primary disease phenotype. *Blood* **109**: 4575–81.

Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, *et al.* (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**: 669–72.

Chan B, Wara D, Bastian J, *et al.* (2005) Long-term efficacy of enzyme replacement therapy for adenosine deaminase (ADA)-deficient severe combined immunodeficiency (SCID). *Clin Immunol* **117**: 133–43.

Colvin GA, Lambert JF, Abedi M, *et al.* (2004) Murine marrow cellularity and the concept of stem cell competition: geographic and quantitative determinants in stem cell biology. *Leukemia* **18**: 575–83.

Davies SM, Kollman C, Anasetti C, *et al.* (2000) Engraftment and survival after unrelated-donor bone marrow transplantation: a report from the National Marrow Donor Program. *Blood* **96**: 4096–102.

Engel BC, Podsakoff GM, Ireland JL, *et al.* (2007) Prolonged pancytopenia in a gene therapy patient with ADA-deficient SCID and trisomy 8 mosaicism: a case report. *Blood* **109**: 503–6.

Fischer A, Le Deist F, Hacein-Bey-Abina S, *et al.* (2005) Severe combined immunodeficiency. A model disease for molecular immunology and therapy. *Immunol Rev* **203**: 98–109.

Gaspar HB, Bjorkegren E, Parsley K, *et al.* (2006) Successful reconstitution of immunity in ADA-SCID by stem cell gene therapy following cessation of PEG-ADA and use of mild preconditioning. *Mol Ther* **14**: 505–13.

Gaspar HB, Parsley KL, Howe S, *et al.* (2004) Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet* **364**: 2181–87.

Giblett ER, Anderson JE, Cohen F, *et al.* (1972) Adenosine-deaminase deficiency in two patients with severely impaired cellular immunity. *Lancet* **2**: 1067–69.

Glass B, Uharek L, Gaska T, *et al.* (1993) The influence of graft-versus-host reactivity, lymphocyte depletion, and cell dose on allogeneic bone marrow engraftment. *Bone Marrow Transplant* **12**(3): S41–S47.

Hacein-Bey-Abina S, Le Deist F, Carlier F, *et al.* (2002) Sustained correction of X-linked severe combined immunodeficiency by *ex vivo* gene therapy. *N Engl J Med* **346**: 1185–93.

Hacein-Bey-Abina S, von Kalle C, Schmidt M, *et al.* (2003a) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* **348**: 255–56.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, *et al.* (2003b) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302**: 415–19.

Haddad E, Landais P, Friedrich W, *et al.* (1998) Long-term immune reconstitution and outcome after HLA-nonidentical T-cell-depleted bone marrow transplantation for severe combined immunodeficiency: a European retrospective study of 116 patients. *Blood* **91**: 3646–53.

Harrison DE, Stone, M, Astle, CM. (1990) Effects of transplantation on the primitive immunohematopoietic stem cell. *J Exp Med* **172**: 431–37.

Hershfield MS. (1995) PEG-ADA: an alternative to haploidentical bone marrow transplantation and an adjunct to gene therapy for adenosine deaminase deficiency. *Hum Mutat* **5**: 107–12.

Hirschhorn R. (1993) Overview of biochemical abnormalities and molecular genetics of adenosine deaminase deficiency. *Pediatr Res* **33**: S35–S41.

Hirschhorn R, Yang DR, Puck JM, *et al.* (1996) Spontaneous *in vivo* reversion to normal of an inherited mutation in a patient with adenosine deaminase deficiency. *Nat Genet* **13**: 290–95.

Hofmeister CC, Zhang J, Knight KL, *et al.* (2007) *Ex vivo* expansion of umbilical cord blood stem cells for transplantation: growing knowledge from the hematopoietic niche. *Bone Marrow Transplant* **39**: 11–23.

Hoogerbrugge PM, van Beusechem VW, Fischer A, *et al.* (1996) Bone marrow gene transfer in three patients with adenosine deaminase deficiency. *Gene Ther* **3**: 179–83.

Ishikawa F, Yasukawa M, Lyons B, *et al.* (2005) Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain (null) mice. *Blood* **106**: 1565–73.

Kawamura N, Ariga T, Ohtsu M, *et al.* (1999) *In vivo* kinetics of transduced cells in peripheral T cell-directed gene therapy: role of CD8+ cells in improved