Haematologica 1999; 84:839-843 original paper

Mobilization and selection of peripheral blood hematopoietic progenitors in children with systemic sclerosis

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ABSTRACT

Background and Objective. **Autologous transplant of lymphocyte-depleted peripheral blood stem cells has been proposed for treatment of patients with severe autoimmune disease. However, until now, no data are available on the safety and feasibility of both stem cell collection and selection in pediatric patients with these disorders. We report on three children affected by systemic sclerosis with lung involvement, who received chemotherapy and granulocyte colony-stimulating factor (G-CSF) to mobilize autologous peripheral blood progenitors.**

Design and Methods. **The priming regimen consisted of cyclophosphamide (CY, 4 g/m^2) and G-CSF (lenograstim, 10 µg/kg/day starting 2 days after cyclophosphamide administration until stem cell collection). Leukapheresis was performed when WBC and CD34$^+$ cell count were at least 2×10^9/L and 0.03×10^9/L, respectively. In the first patient, positive selection of CD34$^+$ cells was performed through the Ceprate SC stem cell concentrator (CellPro, Bothell, WA, USA). In the remaining 2 children, progenitor cells were also purged with negative selection of CD4$^+$ and CD8$^+$ lymphocytes performed by means of the Isolex 300i device (Baxter).**

Results. **All patients tolerated the priming regimen well and did not present any sign of autoimmune disease exacerbation. Collection was successful in all children and the number of CD34$^+$ cells before selection ranged between 10.7×10^6 and 17.6×10^6/kg of patient body weight. The selection of haematopoietic stem cells in the 3 patients resulted in at least 2.6-log T-cell depletion of the cell content, with a recovery of the initial value of CD34$^+$ cells comprised between 21 and 44%. After, a preparative regimen consisting of CY (200 mg/kg over 4 days) and Campath-1 G *in vivo* (10 mg/day for 2 consecutive days), patients were transplanted using cryopreserved lymphocyte-depleted progenitor cells. In all cases, a prompt hematopoietic engraftment was observed.**

Interpretation and Conclusions. **Taken together these data suggest that mobilization, collection and selection of hematopoietic progenitors are safe and feasible in children with autoimmune disease.**

Key words: autoimmune disease, peripheral blood stem cell transplantation, cytokines, T-cell depletion, mobilization

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Systemic sclerosis (SSc) is a rare autoimmune disease (AID) of unknown aetiology; fewer than 10% of all cases have their onset during childhood.[1] The most widely accepted theory of the pathogenesis of the disease considers vascular endothelial injury, recruitment of inflammatory cells, increased fibroblast proliferation and collagen synthesis as the fundamental elements leading to SSc.[2]

The diffuse variant of SSc has been reported to be associated with a 5-year mortality of 40% and pulmonary, cardiac or renal involvement are the main factors influencing patients' prognosis.[3] In particular, pulmonary hypertension and interstitial lung disease are now the two principal causes of death in children with SSc.[1] In fact, currently, no therapy of proven efficacy exists for pulmonary hypertension or interstitial lung disease, whose occurrence predicts for a poor outcome in a short-term period.[4]

Since a substantial immunologic component seems to be implied in the pathogenesis of the disease and some success has been reported after intensive immune suppressive therapy, high dose chemotherapy followed by autologous transplant of mobilized, lymphocyte-depleted, peripheral blood stem cells (PBSC) has been proposed for treatment of patients with the most severe form of this disorder.[5-9] However, no data are available on the safety and feasibility of mobilization, as well as on the efficiency of lymphocyte depletion, in pediatric patients. We report on three children with SSc who underwent hematopoietic stem cell collection and selection after a homogeneous priming regimen with cyclophosphamide (CY) and granulocyte colony-stimulating factor (G-CSF).

Design and Methods

All patients suffered from SSc with diffuse skin involvement, Raynaud's phenomenon and pronounced lung abnormalities, without pulmonary hypertension. In particular, in all cases, pre-mobilization high-resolution CT scan of the chest documented inflammatory alveolitis and signs of lung fibrosis. Lung function tests performed in the 3 patients before mobilization showed mild to moderate restriction and severe impairment of diffusion capacity. None of the children had responded to conventional immunosuppressive treatment, including CY and methotrexate, and, at time of mobilization, they were receiving steroids, which were discontinued starting the priming regimen.

Children underwent the mobilization procedure after approval from the hospital ethic committee and written informed consent from parents had been given. Details on patients' age, sex, disease duration, body weight and estimated blood volume are reported in Table 1.

Peripheral blood progenitors were mobilized using CY given in 1-hour iv infusion at a dose of 4 g/m^2 on day 0, followed by administration of G-CSF (lenograstim, Rhône-Poulenc Rorer), administered at a dose of 10 µg/kg/day, starting on day +2 until PBSC collection. Uromitexan was employed to prevent the occurrence of CY-induced hemorrhagic cystitis. Day of CY administration was taken as day 0.

All children had a double-lumen right atrial central venous line placed. Low microbial diet was initiated after mobilization and, as prophylaxis of infections, children were given ciprofloxacin and fluconazole from admission to recovery of an absolute neutrophil count greater than 0.5×10^9/L. To prevent endogenous reactivation of herpes simplex virus (HSV), children received prophylactic intravenous acyclovir at a dose of 750 mg/m^2 starting 2 days before mobilization until discharge. Leukocyte-poor, PALL-filtered transfusions were employed and all blood products were irradiated to avoid the risk of transfusion-associated graft-versus-host disease. As *Pneumocystis carinii* pneumonia prophylaxis, patients were treated with oral cotrimoxazole. Usually, empirical broad-spectrum antibiotic therapy was started when children became febrile. Toxicity related to the mobilization procedure was graded according to the criteria proposed by Bearman *et al.*[10]

Monoclonal antibodies used in this study for monitoring the number of CD34$^+$ cells in peripheral blood and for evaluating both the recovery of hematopoietic progenitors and the efficiency of T-cell depletion included: anti-CD34-PE (HPCA2) and anti-Leu4 (CD3)-FITC (Becton Dickinson, Mountain View, CA, USA). Phenotypic analysis was performed by means of direct immunofluorescence on a FACScan flow cytometer (Becton Dickinson).

Collection was performed only when WBC and CD34$^+$ cell count were at least 2×10^9/L and 0.03×10^9/L, respectively. Leukapheresis was performed with SPECTRA COBE (Lakewood, Col., USA) version 4.7, processing a minimum of two blood volumes at a mean blood flow rate of 30 mL/min (range 22-35). ACD-A (acid citrate dextrose, formula A) was employed at an anticoagulant/whole blood ratio of 1:12. To prevent or control hypocalcemia, calcium-gluconate was administered at regular intervals (every 30-45 minutes). Continuous monitoring of the patient's vital signs was carried out during the procedure.

Table 1. Patients' characteristics.

	PI	*TA*	*ND*
Age (years)	12	11	9
Sex	Female	Male	Female
Disease duration (years)	7.5	2.5	3.5
Patients' body weight (Kg)	24	30	24
Number of procedures	2	1	2
Day of stem cell collection*	+12 and +13	+10	+11 and +12
Estimated patient blood volume	1.670	2.574	1.742
Processed blood volume per procedure (mL)	3,500	6,500	5,000
Absolute number of CD34$^+$ cells/mL at time of PBSC collection	68 and 77	191	72 and 47

**Starting point is considered the day of cyclophosphamide administration.*

Collected peripheral blood progenitor cells were lymphocyte-depleted via positive selection of CD34$^+$ cells in all children. In detail, in the first patient (PI), the Ceprate SC stem cell concentrator (CellPro Bothell, WA, USA) was utilized. In the remaining 2 children (TA and ND), progenitor cells were also purged with negative selection of CD4$^+$ and CD8$^+$ lymphocytes performed by means of the Isolex 300i device (Baxter). Considering an expected 30% progenitor cell recovery after CD34$^+$ enrichment, we decided to collect at least 6×10^6 CD34$^+$ cells/kg of patient body weight. In fact, this threshold should provide a minimum of 2×10^6 CD34$^+$ cells/kg of patient body weight, a value considered to be sufficient for complete hematopoietic reconstitution.[11]

Controlled freezing of selected PBSC was performed in dimethylsulfoxide and the cells were stored at –180°C until transplantation.

Results

The immediate period after mobilization was substantially uneventful in all patients, who experienced complete recovery of hematopoiesis. In particular, the only observed procedure-related non-hematologic toxicity was grade I-II mucositis. During the period of neutropenia, children had 2 days of fever each. None of the patients experienced exacerbation of SSc fol-

lowing treatment with CY and G-CSF. Time to hospital discharge after mobilization was 16, 14 and 14 days for patients PI, TA and ND, respectively.

The children recovered more than 0.5×10^9/L granulocytes 13, 10 and 10 days after mobilization. Time needed to achieve a platelet count higher than 50×10^9/L for patient PI was day +13, the remaining 2 children (TA and ND) did not experienced a nadir value below this threshold.

Collection of PBSC was well-tolerated and successful in all cases. Two patients required 2 procedures and one needed a single procedure to obtain the target number of $CD34^+$ cells. Details on the number of circulating $CD34^+$ cells at time of collection, day of collection(s) and blood volume processed for each patient are reported in Table 1.

The total number of PBSC collected in the 3 patients was 4.0, 4.6 and 4.9×10^8/kg. Positive selection of $CD34^+$ cells, associated with negative selection of $CD4^+$ and $CD8^+$ cells in the last 2 patients, resulted in at least 2.6 log T-cell depletion of the cell content, with a recovery of the initial value of $CD34^+$ cells ranging between 21 and 44%.

Detailed information on the number of $CD34^+$ cells obtained before and after stem cell selection, percentage of $CD34^+$ cell recovery, number of T-lymphocytes before and after progenitor cell selection and amount of T-cell depletion for each patient is reported in Table 2. Final $CD34^+$ cell purity of cryopreserved PBSC was between 85 and 96%.

All children were transplanted with the selected cryopreserved PBSC after a preparative regimen consisting of CY (200 mg/kg over 4 days) and Campath-1 G *in vivo* (10 mg/day for 2 consecutive days). Viability of selected progenitors after thawing was between 80 and 90% and all patients reached a prompt recovery of hematopoiesis. All children recovered an absolute neutrophil count greater than 0.5×10^9/L on day +11 after transplantation and they did not experience any relevant transplant-related complication. Patients were discharged from the hospital 16, 21 and 18 days after transplantation.

Table 2. Characteristics of progenitor cell mobilization and selection.

	PI	*TA*	*ND*
Number of collected mononuclear cell/kg	4.0×10^8	4.6×10^8	4.9×10^8
$CD34^+$ cells/kg before cell processing	10.7×10^6	17.6×10^6	11.4×10^6
$CD34^+$ cells/kg after cell processing	3.3×10^6	3.7×10^6	5.1×10^6
% of $CD34^+$ cell recovery	31	21	44
$CD3^+$ cells/kg before cell processing	291×10^5	172×10^5	245×10^5
$CD3^+$ cells/kg after cell processing	0.67×10^5	0.016×10^5	0.27×10^5
Log of T-cell depletion	2.64	4.03	2.96

Discussion

The theoretical rationale for using autologous transplant of hematopoietic stem cells in patients with severe AID stems from the following considerations:

i) cytotoxic drugs are able to kill autoreactive lymphocyte populations in a dose-dependent way;[12]
ii) experiments in animal models have documented that high-dose cytoreductive treatment followed by the infusion of syngeneic or even autologous hematopoietic stem cells is effective in inducing remission in naturally occurring or experimentally-induced AID;[13,14]
iii) newly regenerating T and B lymphocytes after chemotherapy can hypothetically be expected to be re-educated to acquire tolerance towards self components, which is lost in AID patients (altered self);
iv) finally, autologous transplantation of hematopoietic progenitors is certainly safer than allograft, which proved to be effective in producing sustained remission and even cure of individuals with hematologic disorders and concurrent AID.[15-17]

Autologous PBSC are rapidly replacing bone marrow cells as a source of hematopoietic progenitors for rescue after high-dose therapy in childhood cancer because of their faster kinetics of engraftment of all hematopoietic lineages.[18-20] The shortened period of cytopenia is expected to reduce transplant-related morbidity and mortality, this consideration being of particular relevance in the medical decision process leading to the use of an autograft in the treatment of children with AID. Thus, information on safety and feasibility of peripheral blood stem cell collection in patients with AID is of paramount importance and our study is the first report specifically addressing these issues in paediatric patients.

Release of large numbers of hematopoietic progenitors into the circulation can be obtained at time of recovery following myelosuppressive chemotherapy and after treatment with hematopoietic growth factors (HGF), administered as single agents or as enhancement of chemotherapy mobilization. A combination of chemotherapy and cytokines has been demonstrated to be associated with a more predictable yield of collection and to provide a greater number of progenitors in comparison with either chemotherapy or HGF alone.[18,19] The number of autologous PBSC required for an optimal hematological recovery is still controversial, this fact partially reflecting difficulties in standardizing the quantitative assay used. A number of 2×10^6 $CD34^+$ cells/kg or $10\text{-}20\times10^4$ granulocyte macrophage progenitors (CFU-GM)/kg is considered to be sufficient for com-

plete hematopoietic reconstitution.[11,18] However, a greater number of CFU-GM or CD34+ cells reinfused, as well as the use of HGF after PBSC transplantation, results in more rapid engraftment, with a consequent reduction of the duration of both neutropenia and thrombocytopenia.[18-20]

On the basis of these considerations, in our children with SSc we chose to take advantage of the synergistic mobilizing effect of myelotoxic drugs and HGF to obtain at least 2×10^6 CD34+ cells/kg after progenitors selection. Since this target threshold was easily achieved in all children, our study indicates that in pediatric patients with AID the capacity to mobilize hematopoietic progenitors is well preserved, probably not being significantly different from that of healthy donors and better than that observed in cancer patients, whose previous treatment with chemotherapy may unfavorably affect PBSC yield. Notably, even though also our patients had previously received MTX and CY as therapy for SSc, this treatment did not influence their mobilizing capacity.

A theoretical concern related to the use of HGF for mobilization, raised by few anecdotal reports,[21,22] is that of a possible flare-up of arthritis and vasculitis, perhaps through leukocyte activation. Our patients did not experience any exacerbation of SSc and we believe that the concomitant administration of CY should spare patients from any possible risk of induction of AID flare-up related to the use of G-CSF. Moreover, it should also be considered that the use of G-CSF during mobilization could offer the advantage of favoring a polarization of regenerating T-lymphocytes towards the production of type-2 cytokines (namely IL-4 and IL-10), which display an anti-inflammatory effect. In fact, in an animal model G-CSF has been demonstrated to induce T-cells towards this polarization, which also proved to be long-lasting.[23] It should be also underlined that, in normal subjects, G-CSF is able to increase the production of two important cytokine antagonists, i.e. soluble TNF-receptor and IL-1 receptor antagonist.[24]

Since T-lymphocytes are implicated in the pathogenesis of several AID, there is an evident theoretical rationale for *ex vivo* and *in vivo* depletion of these cells, in which immunologic memory reside.[8,9,25-27] Our approach proved to be capable of removing around 3-log T cell from the collected stem cells, this leading to the infusion of a number of CD3+ cell lower than 1×10^5/kg. There is no definitive consensus on whether and, if so, to what degree it is necessary to deplete T-cells in patients with AID given autologous PBSC transplant. However, it is reasonable to speculate that if profound destruction of the autoreactive lymphocyte population is mandatory for the success of autograft in these patients, the results of *ex vivo* lymphocyte removal we obtained, coupled with efficient *in vivo* lymphocyte killing by means of pre-transplant preparative therapy, should be sufficient to achieve this goal. The effect of this PBSC manipulation in terms of immune reconstitution and ability to mount efficient immune defences against infection after autograft is yet to be evaluated in further clinical studies.

In summary, our results indicate that the combination of CY and G-CSF is safe and effective for mobilizing a huge number of PBSC in children with SSc. Positive selection of CD34+ cells coupled with negative selection of lymphocytes allow both at least a 3-log *ex vivo* T-cell depletion and a 30% recovery of initial number of CD34+ progenitors to be obtained. The clinical efficacy of this approach will be proved in future studies on the role of autografting in pediatric patients with AID.

Contributions and Acknowledgements

FL directed the execution of transplants, designed the study and wrote the paper. CP and LT performed stem cell collections, as well as, together with DM and RM, CD34+ cell selection. GG, EG, MLM and PDS participated in the mobilization procedures and in the clinical care of the patients. FDB, AR and AM were responsible for the initial diagnosis and the whole caring process of the patients. The last author (AM) contributed to the study design.

We are indebted to Dr. Patrizia Comoli, Piero De Stefano, Stefania Viola and Marco Zecca for their valuable help.

Funding

This work was partly supported by grants from Associazione Italiana Ricerca sul Cancro (AIRC) and IRCCS Policlinico San Matteo, Pavia to FL and RM.

Disclosures

Conflict of interest: none

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received February 12, 1999; accepted May 7, 1999.

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