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Matched Unrelated Hematopoietic Stem Cell Transplantation Using Selected CD34$^+$ Cells in Fanconi's Anemia: Experience of One Center

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In order to prevent Graft versus Host Disease (GvHD) in Matched Unrelated Donor Hematopoietic Stem Cell Transplantation (MUD-HSCT) in Fanconi's Anemia (FA), we report the results of 15 children transplanted by CD34$^+$ positive cells selected by immunomagnetic method isolex/isolex 300i (Baxter Healthcare Corp. USA) in the period between April 1996 and September 1999. Methods: The median total nucleated cells infused 3×10^8E kg^{-1} (0, 65- 3, 95), CD34 cells 2.12×10^6E kg^{-1} (0.7-5.8), CD3 cells 2.56×10^6E kg^{-1} (0.001-0.5). Median follow-up is 131 days (34-3022) till 06/2005. All patients achieved engraftment except 2 and another 2 rejected early post transplant. Acute GVHD > grade II and extensive chronic GVHD occurred in 4 cases and one case, respectively. Ten/Fifteen patients died. Transplant related mortality in 8 patients (graft failure n = 4, fungal infection n = 3 and GVHD n = 1), 2ry leukemia and relapse of initial disease in one patient, respectively. Five cases are alive and well 6 years at least post transplant. Positive selection of CD34 cells minimizes GVHD in Fanconi's anemia patients and gives acceptable survival. However, graft failure remains the main obstacle.

Key words: Fanconi's anemia, hematopoietic stem cell transplantation, CD34$^+$ cells

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INTRODUCTION

Fanconi's anemia is an autosomal recessive heterogeneous disorder characterized by progressive pancytopenia, diverse congenital abnormalities and increased predisposition to malignancies (D'Andrea, 2003; Mathew, 2006).

However, the permanent cure is only provided by Allo HSCT to restore normal hematopoiesis (Gluckman *et al*., 1995; Guardiola, 2000). Five year survival can reach more than 70% in the presence of an identical sibling donor (Gluckman, 1996; Socie *et al*., 1998). Alternatively, MUD can be proposed, but with previous disappointing results as 3 year overall survival reaching 33% (Guardiola *et al*., 2000; Wagner *et al*., 2004). As Fanconi's anemia cells show a high chromosomal breakage, the exposure to immune competent T-lymphocytes and thus graft versus host disease would be to toxic (Guardiola *et al*., 2004). Previous trials of *in vitro* TCD by negative selection techniques, although markedly diminished GVHD, they were followed by increased incidence of graft rejection or severe infections (Ash *et al*., 1990). We report the results of 15 FA children, undergoing MUD HSCT, after positive selection of $CD34^+$ cells capable of lympho-hematopoiesis for rapid engraftment using the Isolex 300 i (Baxter Healthcare Corp. USA) to control T-cell depletion with a permissible T-cell infusion minimizing GVHD incidence.

Present objectives were to evaluate GVHD and engraftment incidence and long term outcome.

MATERIALS AND METHODS

The study was prospectively conducted in the bone marrow transplantation unit, Saint-Louis hospital, Paris, France in the period between April 1996 and September 1999. Diagnosis of FA depended on clinical picture as well as chromosomal breakage tests. The patients characteristic, conditioning regimen and cell therapy are shown in Table 1.

We have transplanted 15 Fanconi's anemia children showing bone marrow failure syndrome, 5 males and 10 females, median age 11 years (5-12). The protocol was decided by the hematology federation, hospital Saint-Louis and approved by the ethics Committee of experts. All parents gave written informed consent. The median delay between diagnosis and transplantation was 672 days (237-5443). MUD bone marrow graft with HLA identical on class I A, B serology and Class II DR generic and specific alleles by molecular biology techniques was used. Conditioning regimen consisted of EDX 40 mg kg^{-1} total dose, fractionated total body irradiation 4.5 Gy and ATG 12.5 mg kg^{-1} total dose. GVHD prophylaxis was ciclosporin A 3 mg kg^{-1} which could be substituted for corticosteroids 1 mg kg^{-1} in case of renal toxicity. The bone marrow harvesting technique was described by Thomas and Storb (1970). Stem cells source was subjected to $CD34^+$ cells positive selection by the Baxter Biotech Isolex *in vitro* prophylaxis using 9.C5 anti-CD34 antibody, immunomagnetic bead technique. Baxter Immunotherapy division provided the device and reagents. An enzymatic release step was used to separate the positively selected cells from the immunomagnetic beads. The procedure was done by the fully automated immunomagnetic beads system Isolex 300i except the first 5 candidates: Semi automated Isolex 300 SA (Civin, 1996). Administration of $CD34^+$ cells immediately after processing over 15 min through the central venous catheter without special measures. The non-CD34 cell fraction was also sterilely collected, cryopreserved and

Table 1: Patients characteristics, conditioning regimen and cell therapy

UPN	Diagnosis	Age	Gender	Transplant date	Pretransplant transfusion	Serology HLA A,B, DR Gen./sp.	CMV R/D	Conditioning EDX/TBI/ATG	TNC 10^8 kg^{-1}	CD34 10^6 kg^{-1}	CD3 10^6 kg^{-1}
1	FA	12	F	04/96	<20	Identical	-/+	+	3.80	3.50	0.19
2	FA	6	F	11/96	<20	Identical	-/+	+	3.00	2.12	0.07
3	FA	12	F	01/97	<20	Identical	-/-	+	1.30	1.25	0.50
4	FA	5	M	02/97	<20	Identical	-/-	+	3.00	2.44	0.03
5	FA	12	M	04/97	>20	Identical	+/+	+	4.10	3.63	0.16
6	FA	13	F	09/97	>20	Identical	+/-	+	4.30	3.95	0.08
7	FA	11	F	10/97	<20	Identical	-/-	+	0.70	0.65	0.01
8	FA	9	F	11/97	<20	Identical	-/+	+	3.00	2.93	0.03
9	FA	15	M	11/97	<20	Identical	-/-	+	1.00	1.18	0.01
10	FA	8	M	06/98	<20	Identical	-/-	+	2.00	1.64	0.08
11	FA	12	M	08/98	<20	Identical	+/+	+	1.76	1.66	0.001
12	FA	8	F	10/98	<20	Identical	-/-	+	1.00	0.79	0.12
13	FA	12	F	05/99	<20	Identical	-/-	+	2.20	2.12	0.04
14	FA	8	F	05/99	<20	Identical	+/+	+	3.70	3.64	0.06
15	FA	11	F	09/99	<20	Identical	+/+	+	1.70	1.66	0.01

ATG: Anti-thymocyte Globulin, CD: Cluster of Differentiation, CMV: Cytomegalovirus, D: Donor, EDX: Cyclophosphamide, FA: Fanconi Anemia, F: Female, Gen: Generic, M: Male, R: Recipient, Sp: Specific, TBI: Total Body Irradiation, TNC: Total Nucleated Cells, (-/+): Negative/Positive, +: Yes

stored in bags at a concentration of 1×10^7 $CD3^+$ cells kg^{-1} as additional safety backup. The median total nucleated cells infused 2.2×10^8 kg^{-1} of recipient body weight (0.7- 4.3), CD34 cells 2.12×10^6 kg^{-1} (0.7- 4), CD3 cells 0.07×10^6 kg^{-1} (0.001-0.5). The criteria of hematopoietic reconstitution depended on neutrophil count > 500 P mm^3 for 3 consecutive days with and or platelet count > 20000 for one week untransfused with 60 and 180 days cutoff date for neutrophils (PNN) and platelet engraftment, respectively. Glucksberg and IBMTR scores were used to evaluate graft host disease (Glucksberg *et al*., 1974; Martino *et al*., 1999). TRM was defined as mortality occurring within 3 months of transplant not related to initial disease progression.

RESULTS

The median follow up is 131 days (19-3022), from April 1996 to June 2005. The results are shown in Table 2 and 3.

Engraftment and graft failure: All patients achieved engraftment except two: (UPN7 and UPN12) both failed to achieve both PNN and platelet engraftment and were subjected to a 2nd allograft. However out of the remaining 13 patients achieving PNN engraftment, 5 cases did not show platelet engraftment as 2 of them presented with early graft rejection (UPN 4 and 10) and one showed late relapse of the initial disease and disease progression (UPN13). While the remaining 2 died from TRM before reaching the estimated deadline for platelet engraftment (UPN 6 and 11). The median duration of PNN engraftment was 15 days (10-20) and that to reach platelet engraftment was 38.5 days (13-43). GVHD: Acute > grade II and chronic extensive GVHD occurred in 4 and 2 children, respectively.

Viral infections: No CMV disease took place, other viral infections including herpes simplex or zoster occurred but of favorable outcome. JCBK virus was detected in 2 children presenting hemorrhagic cystitis.

Fungal: Aspergillosis was of fatal outcome in the 4 affected patients. However, candidemia either albicans or Krusei occurring in 4 patients responded to treatment.

Bacterial: Bacterial infections were exclusively in the form of septicemia but of favorable outcome.

2ry malignancies and disease progression: Myelodysplastic syndrome (MDS) with excess blasts occurred in one patient 5 years post transplant while another one showed lymphoproliferative syndrome following EBV reactivation 2 years post transplant responding initially to anti CD20 and both died of disease progression.

Long term complications: Long term follow up revealed Rheumato-orthopedic problems in 3 patients and endocrine dysfunction, (delayed puberty n = 1 and hypothyroidism associated to hypogonadism n = 1.

Outcome and survival: Five patients out of 15 are alive and well at least 6 years post transplant. Five patients died of TRM: Aspergillosis systemic FI n = 3, AGVHD n = 1, Graft failure with aspergillosis FI n = 1. The remaining 5 patients underwent 2nd transplant from the same donor without CD34 positive selection either for graft failure n = 3 (non engraftment n = 2, early rejection n = 1), or relapse of initial disease n = 2 associated to 2^{ry} MDS with excess blasts in one of both, but all died following the 2nd transplant.

Table 2: Results of graft versus host disease, engraftment, rejection, late marrow failure

UPN	GVHD prophylaxis	*AGVHD skin	AGVHD GIT	AGVHD liver	Grade	Extensive CGVHD	Engraft	Rejection	TTT	PNN** > 500	platelets** >20000	2 ry marrow failure
1	CSA	0	0	0	0	0	+	0		14	38	Malignancy
2	CSA/PRED	0	0	4	IV	M.M+Liver	+	0		16	39	0
3	CSA/PRED	0	0	0	0	0	+	0		18	41	0
4	CSA/PRED	0	0	0	0	0	+ Day 18	Yes D27	2^n HSCT	20	NR	0
5	CSA/PRED	0	0	0	0	B.O	+	0		12	40	0
6	CSA/PRED	0	0	0	0	NR	+	0		13	NR	0
7	CSA	0	0	0	0	NR	No	0	2^n HSCT	NR	NR	0
8	CSA/PRED	0	0	0	0	0	+	0		10	35	0
9	CSA/PRED	3	2	4	IV	0	+	0		16	43	0
10	CSA/PRED	0	0	0	0	NR	+Day 15	Yes D45	No TTT	15	NR	0
11	CSA/PRED	0	0	3	III	0	+	0		17	NR	0
12	CSA/PRED	0	0	0	0	NR	No	0	2^n HSCT	NR	NR	0
13	CSA	2	2	1	III	0	+	0		14	NR	Malignancy
14	CSA	0	0	0	0	0	+	0		11	13	0
15	CSA	0	0	0	0	0	+	0		16	33	0

*: Staging, **: Duration (days), AGVHD: Acute Graft Versus Host Disease, BO: Bronchiolitis Obliterans, CGVHD: Chronic Graft Versus Host Disease, CSA: Cyclosporin A, PRED: Prednisone, MM: Mucous Membrane, NR: Not Reached, TTT: Treatment

Table 3: Results of infections outcome and mortality

UPN	CMV disease	Other viral	Bacteria	Candidiasis	Aspergillosis	Other fungal	Other complications	2 ry MF malignancy	TTT	Dead	Cause	Cause TRM 1st transplant
1	0	Herpes Zoster	0	0	0	0	0	MDS	2nd HSCT	Yes 5 years	2ry malignancy	
2	0	0	SCN,	Albicans Serratia	CNS	0	0	0	0	Yes	TRM	Aspergillosis
3	0	HSV	SCN	0	0	0	0	0	0	No	0	0
4	0	0	SCN	Krusei	0	0	0	0	0	Yes D 120	TRM 2nd HSCT	Graft falure
5	0	JCBK	SCN, PCN	Krusei	0	T. Glabrata	Monoarthritis	0	0	No	0	0
6	0	0	0	0	Lung + CNS	0	0	NR	0	Yes Day 19	TRM	Aspergillosis
7	0	0	SCN	0	0	0	HC	0	0	Yes D 100	TRM 2nd HSCT	Graft failure
8	0	HSV keratitis	0	0	0	0	0	0	0	No	0	0
9	0	0	0	0	0	0	HC	0	0	Yes	TRM	GVHD
10	0	0	SA	0	Lung+ CNS	0	0	0	0	Yes D 60	TRM	Graft failure Aspergillosis
11	0	0	EC fecium, SA	0	Lung +CNS	0	0	0	0	Yes Day 30	TRM	Aspergillosis
12	0	0	BGN	Krusei	0	0	ARDS	0	0	Yes D 100	TRM 2nd HSCT	Graft failure
13	0	EBV	0	0	0	0	Osteochondritis LPS	Relapse	2nd HSCT	Yes 2 years	Relapse	
14	0	0	0	0	0	0	ITP, DGD Delay puberty	0	0	No	0	0
15	0	JCBK	EC fecium	0	0	0	IP, HC, VZV HG +HT Osteopenia	0	0	No	0	0

ACV: Acyclovir, ARDS: Acute Respiratory Distress Syndrome, CNS: Central Nervous System, D: Day, DGD: Delay Growth and Development, EC: Enterococcus, EBV: Epstein Barr Virus, FI: Fungal Infection, HG: Hypogonadism, HSCT: Hematopoietic Stem Cell Transplantation, HSV: Herpes Simplex Virus, Hgc: Hemorrhagic, HC: Hemorrhagic Cystitis IP: Interstitial Pneumonitis, ITP: Idiopathic Thrombocytopenic Purpura, JCBK: JCBK Virus, LPS: Lymphoprolifferative Syndrome, MDS: Myelodysplastic Syndrome, MF: Marrow Failure, NR: Not Reached, PCN: Pseudomonas, SA: Staphylococcus Aureus, SCN: Staphylococcus Coagulase Negative, HT: Hypothyroidism, T: Torulopsis Glabrata, TRM: Transplant Related Mortality, VZV: Varicella Zoster Virus

DISCUSSION

This is a descriptive study of a phase I/II trial in matched unrelated HSCT using the positive selection of $CD34^+$ cells as a stem cell source. This trial was undergone in a determined period for Fanconi anemia patients. The main target was to assure rapid engraftment ($CD34^+$ and $CD3^+$ dependent) and to minimize as much as possible the risk GVHD ($CD3^+$ dependent) considered to be a high risk factor for those vulnerable patients. Achieving the previous 2 goals would minimize transplant related mortality and thus increases survival. Although the limited number of patients represents an obstacle for statistically significant results, many clinically significant end points have been observed. In this study, failure of engraftment was associated with a low number of TNC and CD34 cells 1×10^8E kg^{-1} and 0.7×10^6E kg^{-1}, respectively even with a higher $CD3^+$. We can see that about half of our cases received less than 2×10^6E CD34 kg^{-1} and nearly all graft failures were in that group. This fact could be related to lower purity and CD34 cells recovery in the selection technique as we targeted > 3×10^6E CD34 cells kg^{-1}. Even with a low CD3 cells, solid engraftment was guaranteed by a satisfactory CD34 cell dose. We can notice that graft failure was associated with higher CD3 depletion only when associated with lower $CD34^+$ cell dose. From here, it is obvious that high incidence of graft failure is still present and responsible of deaths. In the literature in FA transplantation, the probability of engraftment is as low as 63-75% in T-cell depleted marrow (MacMillan *et al*., 2000). It starts from 82% in non T-cell depleted MUD transplants (Guardiola *et al*., 2000). Partial TCD has been found to be associated with a lower risk graft failure than total T-cell depletion and higher than non-TCD (Marmont *et al*., 1991). Although the optimum number of T cells left in the graft to prevent GVHD and to assure engraftment is unknown, a threshold of 0.2×10^6E kg^{-1} of CD3 (as in agreement with results in murine models), seems to be satisfactory (Urbano-Ispizua *et al*., 2001). However, the

number of $CD34^+$ cells is critical for a successful engraftment and is considered even more important than the number of T lymphocytes when TCD is used (Uharek *et al*., 1994). We stress on the dual beneficial role of CD34 and CD3 for successful long-term engraftment. It remains to detect the optimum cell dose: $CD34^+$ and CD3. In order to control GVHD by *in vitro* T-cell depletion using the positive selection $CD34^+$, the median of T-cells infused was CD3 cells 2.56×10^6E kg^{-1} (0.001-0.5) corresponding to 2-3 log-subtractions of T-cells which proves the efficiency of the technique. It has been demonstrated that 1 million of CD3 cells kg^{-1} was sufficient to induce AGVH reaction (Martinez, 1999). In this study, in the majority of cases, injected CD3 cells was far less than this figure. This corresponded to a remarkably lesser incidence of AGVHD. Only 27% of patients presented with grade III/IV AGVHD and only 2 patients out of 15 showed severe CGVHD. Boyer *et al.* demonstrated that transplantation of $CD34^+$ selected cells from alternative donors resulted in a very low risk of GVHD in children with Fanconi's anemia (Boyer *et al*., 2003). Previously, it was demonstrated that the probability of grade III-IV AGVHD declined from 50% in non T-cell depleted MUD to 8% in T-cell depleted MUD (Guardiola *et al*., 2000). In another study the incidence of grade II and III acute GVHD was 28 and 8%, respectively and 12.5% in chronic GVHD in HLA matched related donors (Dufour *et al*., 2001), while the probability of grade II-IV AGVHD was 32 and 0% in CGVHD in T-cell depleted MUD (MacMillan *et al*., 2000). As a result of T-cell depletion, there is a slow recovery of $CD4^+$ lymphocytes during the first 6 months post transplant, which could be related to a probability of severe infections (Engelhard, 1998; Sehn *et al*., 1999). During the last few years, the incidence of candidiasis and aspergillosis infections has been increasing (Balduzzi *et al*., 1995). Kruger showed that 24 to 30% of isolated pathogens were fungi (Kruger *et al*., 1999). In this study, aspergillosis infection was responsible of dismal outcome. Viral infections are important in SCT patients due to changing immune defects in the patient's immune status The rate of CMV disease is increased and could occur up to more than 2 months post transplant (Boeckh *et al*., 2003). In our center, there was no incidence of CMV disease due to prompt precautions and early pre-emptive therapy but only slight viral infections. Following transplantation, bacterial pathogens are gram-negative and positive (Elishoov *et al*., 1998) and may be responsible of septic shock (Kruger *et al*., 1999). In our centre, more than half of the patients presented with severe infection in the form of septicemia and or pneumonia but they were of favourable outcome. Soçié *et al.* (2000) showed an increased risk of solid cancers and lymphoproliferative disease (LPD) after allogeneic Bone Marrow Transplantation (BMT) for childhood leukaemia, (Soçié *et al*., 2000). In this study one patient presented with LPD, 2 years post transplant probably due to persistent immuno-suppression and another patient manifested myelodysplastic syndrome and leukemia, this could be related to recurrence of initial disease. So transplant recipients, especially those given radiation, should be monitored closely for second cancers. Long term complications as endocrinal dysfunction in the form of hypothyroidism, hypoparathyroidism and hypogonadism are routinely seen as long term effect of allogeneic HSCT (Ghavamzadeh *et al*., 2003) due to prolonged exposure to chemo radiotherapy and other catabolic agents. In our study, 4 patients out of the 5 survivors showed long term complications. Such a fact seems to be logic with long term follow up reaching up to 8 ½ after transplantation. The one year probability of survival in MUD-HSCT with counter flow elutriation T cell depletion in 29 FA patients reached 34% (MacMillan *et al*., 2000). This is similar to the 33% 3 year probability of survival of 69 patients undergoing MUD HSCT with no significant outcome improvement by T-cell depletion (Guardiola *et al*., 2000). In our study, the 6 year survival is 33.5% with a minimal role of GVHD in mortality. But the gained bonus of this decreased incidence of GVHD factor in survival will be lost by a higher risk of graft failure, immunological deficiency responsible for severe fungal infections. The survived patients at least 6 years post transplant are considered to be cured. Positive selection MUD-HSCT is feasible in transplantation of vulnerable Fanconi's patients. However we need more definition of the optimum $CD34^+$ and $CD3^+$ cell doses equilibrium that assure hematopoietic reconstitution with lesser lethal GvHD incidence. As we were confronted with a limited CD34 cell recovery related to the technique, we suggest a prospective study utilising a more powerful CD34 selection technique in term of purity and cell recovery to assure better engraftment.

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