In Vitro Mesenchymal Progenitor Cell Expansion is a Predictor of Transplant-related Mortality and acute GvHD III-IV After Bone Marrow Transplantation in Univariate Analysis: A Large Single-Center Experience

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Summary: Mesenchymal stromal cells (MSCs) are multipotent stem cells able to differentiate into mesenchymal origin tissue and support the growth of hematopoietic stem cells. In order to understand the role of MSCs infused in bone marrow grafts, 53 consecutive patients were analyzed for engraftment, acute and chronic graft-versus-host disease (GvHD), transplant-related mortality (TRM), relapse incidence, and overall survival. The MSC content was measured as MSC expansion at the second passage. When in vitro–expanded MSC (cumulative population doubling at second passage, cPDp2) values were stratified according to the median value (2.2-fold increase), the univariate analysis showed a significant difference in TRM (23% vs. 3.8%, $P = 0.05$.) and in acute GvHD III-IV incidence (12% vs. 4%, $P = 0.04$), while the multivariate analysis did not confirm its independent role. No clinical parameters in donors and recipients were identified as predictors of cPDp2 expansion. Our study suggests a role for short-term ex vivo–expanded MSCs in reduced aGVHD III-IV incidence and TRM in univariate analysis. A multicenter, larger study is warranted to confirm these data.

Key Words: mesenchymal stem cells, bone marrow transplantation, acute GvHD, transplant-related mortality

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Allogeneic hematopoietic stem cell transplantation (HSCT) is the treatment of choice for many malignant and nonmalignant disorders.[1,2]

Mesenchymal stromal cell (MSCs), also known as mesenchymal stromal/stem cells, are non-hematopoietic stem cells (HSCs) that were originally defined as self-renewing, multipotent progenitor cells with multilineage potential to differentiate into other types of cells of mesodermal origin.[3] These cells also provide support for the growth and differentiation of hematopoietic progenitor cells in bone marrow (BM) microenvironments and, in animal models, promote engraftment of hematopoietic cells.[4,5] In coculture experiments with allogeneic lymphocytes, MSCs do not induce lymphocyte proliferation, interferon-γ production, or an upregulation of activation markers.[6,7] Furthermore, MSCs suppress proliferation of activated lymphocytes in vitro in a dose-dependent, non–human leukocyte antigen (HLA)-restricted, manner.[6,8] It has also been shown that stromal cells may be damaged by chemo-radiotherapy before HSCT,[9] and, from a clinical point of view, data on previous studies showed how the add back of stromal cells entrapped in filters during HSCT provided an advantage in terms of reduced graft-versus-host disease (GvHD) and lower transplant-related mortality (TRM).[10]

To date, the human BM fibroblast colony–forming units (CFU-F) and the adherent ratio are the easiest parameters of MSC content in the graft, but the role of MSCs to (1) expand in vivo, (2) maintain stemness in vivo and later be able to differentiate into a committed lineage, and (3) survive and engraft in the recipient, is under investigation. As the role of MSCs transplanted in the BM graft is still not fully understood, and the role of CFU-F is still under debate, we decided to study the in vitro expansion ability of MSCs and, then, to compare their role in reducing transplant toxicity, and improving survival.

PATIENTS AND METHODS

Patients

A retrospective study was carried out of 53 patients, who were treated by allogeneic BM transplantation at our center—Regina Margherita Children Hospital, Pediatric Onco-Hematology and Stem Cell Transplant Division—between March 2009 and October 2013. Allogeneic donor BM samples were used for MSC isolation and expansion. The patients'parents/legal representative signed the informed consent. The study was conducted in compliance with the principles of the Declaration of Helsinki.

BM Collection and Infusion

HSCs from BM were infused in our patients from 22 unrelated and 31 related donors. The number of total nucleated cells (TNC), $CD34^+$ cells, $CD3^+$ cells, colony-forming unit-granulocyte monocyte (CFU-GM), burst forming unit-erythroid (BFU-E), and long-term culture-initiating cells (LTC-IC) present in the BM collection were

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analyzed, as previously described,[11] and calculated per patient Kg.

MSC Preparation and In Vitro Expansion

The whole BM sample was directly plated in alpha-minimum essential media (Sigma-Aldrich, LTO Irvine, Ayrshire, UK) containing 10% fetal bovine serum (Sigma-Aldrich) at a seeding density of 10,000 cells/cm^2. After 7 days, the nonadherent cells were removed and discarded. The adherent cells were refed every 5 to 7 days and, when confluence was reached, the cells were detached and replated for a further 3 to 5 passages at 1000 cells/cm^2, as previously described.[12] We considered MSCs at passage 1 (P1) as the initial time-point when the cells were harvested and replated. We considered this value as an index to quantify stromal progenitors. The same cellular and culture conditions were maintained by the cellular plating of whole BM and during the expansion process. The following passages were coded with increasing numbers: P2, P3, etc. To evaluate cellular growth, the cell growth rate in terms of population doubling (PD) was computed using the formula log N/log 2, where N indicated the rate (the cell number of the detached cells divided by the initial number of seeded cells) and the expansion in terms of cumulative PD (cPD). The cells were characterized as indicated by the International Society for Cellular Therapy,[13] and minimal criteria were met for them to be defined as MSCs[12,14]

In particular, at each passage, the cells were analyzed for viability, immunophenotype, and proliferative potential, and, also, they were differentiated into osteoblasts, chondroblasts, and adipocytes, as previously reported.[12,14]

Statistical Analysis and Methods

The primary endpoint of this study was to evaluate the role of MSCs in reducing TRM, while the secondary endpoints were to evaluate (i) neutrophil and platelet engraftment, (ii) GvHD II-IV cumulative incidence, (ii) acute GVHD III-IV cumulative incidence, (iv) chronic GvHD cumulative incidence, (iv) 3-year relapse incidence (RI), and (v) 3-year overall survival (OS). The RI was calculated for malignancies only.

Absolute neutrophil count (ANC) engraftment was defined as the first of 3 consecutive days of ANC $\geq 0.5\times10^9$/L, while platelet (PLT) engraftment was defined as the first of 3 consecutive days with PLT $\geq 50\times10^9$/L without transfusion. Acute and chronic GvHD were classified according to the Seattle criteria.[15,16] TRM was defined as the probability of dying without recurrence. Relapse was considered the competitive event to compute TRM. RI was defined as the probability of disease recurrence. OS was defined as the probability of survival, irrespective of the disease state. Graft failure was considered as a relapse for nonmalignant disease, while for malignancies, graft failure patients were censored. Patients were stratified according to the median value, and each outcome was calculated according to these subgroups.

The acute GvHD II-IV, acute GvHD III-IV, chronic GvHD, TRM, and RI cumulative curves were calculated by NCSS software, while the statistical differences were calculated by the Gray test using the R-package.[17] The OS was calculated by Kaplan-Meier statistics[18]; the log-rank test[19,20] was used to calculate the *P*-values. A *P*-value below 0.05 was considered as statistically significant.

RESULTS

A total of 53 consecutive patients underwent HSCT from both related (31, 58%) and unrelated donors (22, 41%). The median age at HSCT was 8.6 years (0.6 to 24.8 y), and the main indication for HSCT was acute leukemia (32 patients, 60%). Ten patients underwent HSCT for nonmalignant disease (19%). Ten patients received HSCT in first complete remission (CR1, 19%), sixteen patients in CR2 (30%), and 3 patients in CR3 (6%). Forty patients (75%) received a myeloablative conditioning regimen; the majority of donors were male individuals (37, 75%), and the median donor age was 25 years (3 to 41 y). See Table 1 for details.

A median of 5.1×10^8 TNC/kg (range: 1.7 to 15.8), 5.8×10^6 CD34$^+$/Kg (range: 1.2 to 16.7), and 53.1×10^6 CD3$^+$/Kg (13 to 177.2) were infused.

Isolation and Analysis of MSCs

The time and the number of passages of MSC expansion was variable among patients; however, we isolated and

TABLE 1. Patients' Characteristics

	Patient Characteristics	N = 53 (100%)
Sex M/F		30/23
Patient age (y)		8.6 (0.6-24.8)
Disease	AML	10 (19)
	ALL	22 (41)
	Non-Hodgkin lymphoma	2 (4)
	Nonmalignant disease	10 (19)
	Hodgkin lymphoma	1 (2)
	Myelodysplastic symdrome	3 (6)
	Neuroblastoma	5 (9)
Disease status at HSCT		
	CR1	10 (19)
	CR2	16 (30)
	CR3	3 (6)
	PR	5 (9)
	Untreated/present disease	19 (36)
CMV	Negative	5 (9)
	Positive	49 (92)
HLA match	Identical sibling	21 (40)
	Unrelated 10/10	14 (26)
	Unrelated $\leq$9/10	18 (34)
Conditioning	Myeloablative	40 (75)
	Nonmyeloablative	13 (24)
GvHD prophylaxis	Cy-A	10 (19)
	Cy-A+MTX	12 (23)
	Cy-A+MTX+ATG	31 (58)
Donor age (y)		25 (4-57)
Sex mismatch		13 (24)
		40 (75)
TNC ($\times10^8$)/kg		5.1 (1.7-15.8)
CD34$^+$ ($\times10^6$)/kg		5.8 (1.2-16.7)
CD3$^+$ ($\times10^6$)/kg		53.1 (13-177.2)
CFU-GM ($\times10^4$)kg		19.9 (3.5-103.4)
BFU-E ($\times10^4$)/kg		16.1 (3.9-59.9)
LTC-IC ($\times10^2$)/kg		11.1 (0.6-144.5)

ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; ATG, polyclonal anti-T globulin; BFU-E, burst forming unit-erythroid; CFU-GM, colony-forming unit-granulocyte monocyte; CMV, cytomegalovirus; CR1, first complete remission; CR2, second complete remission; CR3, third complete remission; Cy-A, Cyclosporine-A; F, female; GvHD, graft versus host disease; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; LTC-IC, long-term culture-initiating cells; M, male; MTX, Methotrexate; PR, partial remission; TNC, total nucleated cells.

TABLE 2. Mesenchymal Stromal Cells Characteristics: Immunophenotype Analyzed by Cytofluorimetric Analysis

	p1	p2
$C90^+$	> 90% (91%-100%)	> 90% (91%-100%)
$CD73^+$	> 90% (91%-100%)	> 90% (91%-100%)
$CD105^+$	> 90% (91%-100%)	> 90% (91%-100%)
$CD146^+$	> 80% (50%-99%)	> 80% (33%-98%)
$HLA\text{-}DR^+$	< 10% (0%-8%)	< 2% (0%-1%)
$CD14^+/34^+/45^+/19^+$	< 10% (0%-8%)	< 2% (0%-1%)

expanded MSCs until the second passage from all the patients, with a median time for the P1 of 15 days (range: 11 to 20 d), for the P2 of 28 days (range: 18 to 40 d), and for P3 of 38 days (range: 28 to 50 d). Isolated MSCs were negative for all hematopoietic antigens and for HLA-DR, and expressed > 90% of CD90, CD73, CD105, and CD146, as shown in the Table 2. Isolated MSCs were also able to differentiate into osteoblasts, adypocytes, and chondrocytes.[12,14]

As all patients' MSCs were cultured until the second passage, we considered cPDp2 as a value to analyze the MSCs. As shown in Table 3, the ANC and PLT engraftment speed was not affected by the MSC growth. A trend for better OS was observed for patients having higher cPDp2 values ($P > 0.05$). We observed a reduced TRM for patients having an HSCT content of cPDp2 above the 50th percentile ($P = 0.05$). A whisker plot graph shows the cPDp2 growth differences among TRM and non-TRM patients (Fig. 1). Acute GvHD II-IV was not significantly different among the groups (P = NS), while severe aGvHD III-IV was reduced in patients having a higher expansion of MSC ($P = 0.04$.). The cGvHD incidence and the RI did not differ among the groups (P = NS).

Multivariate Analysis

The multivariate model shows that cPDp2 growth values do not play a significant independent role in TRM risk (hazard ratio [HR], 0.99; 95% confidence interval [CI], 0.98-1.01), while the HLA match (unrelated donor with HLA match below 9/10 [HR, 3.23; 95% CI, 0.33-32.2]) and the conditioning regimen (myeloablative [HR, 2.19; 95% CI, 0.22-22.1]) seem to increase the risk of TRM, even if the small numbers do not allow to reach the statistical significance. The GvHD III-IV risk shows a similar trend, even if the reduced number of the sample makes the interpretation even more uncertain.

TABLE 3. Outcome of Patients According to cPDp2 Count

	CPDp2 Above the Median	CPDp2 Below the Median	*P*
ANC	20 (11-32)	20.5 (14-32)	NS
PLT	23 (12-191)	29 (21-125)	NS
TRM	3.8% (0-26)	23% (11-46)	0.05
RI	38% (23-62)	23% (11-46)	NS
GvHD II-IV	32% (18-56)	36% (21-60)	NS
GvHD III-IV	4% (0-27)	12% (4-34)	0.04
Chronic GvHD	21% (10-47)	23% (10-49)	NS
OS	60% (41-79)	54% (34-74)	NS

ANC indicates absolute neutrophil count; GvHD, graft-versus-host disease; NS, not significanct; OS, overall survival; PLT, platelets; RI, relapse incidence; TRM, transplant-related mortality.

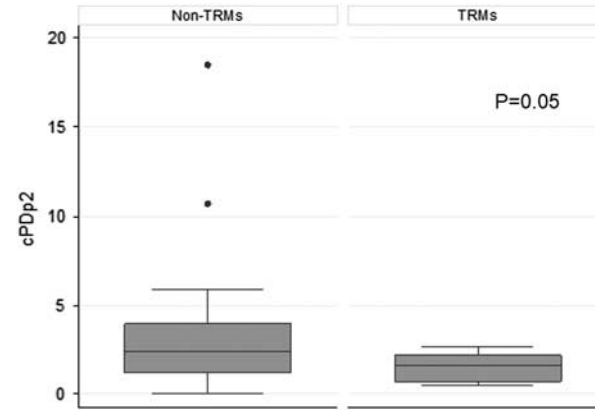

FIGURE 1. Whisker plot distribution of cPDp2 growth among transplant-related mortality (TRM) and non-TRM patients.

Factors Affecting cPDp2 Growth

When we analyzed factors to assess a predictive role for higher cPDp2 content, no significant effects for the patient and donor age, sex, type of disease, conditioning regimen, TNC dose, $CD34^+$ dose, or $CD3^+$ dose were observed; in particular, no significant relation between donor age and expansion potential was observed.

Causes of Death

Five patients having higher cPDp2 growth died. Two patients died from disease progression (40%), 1 patient from posttransplant lymphoproliferative disease (20%), 1 from acute respiratory distress syndrome (20%), and 1 from acute GvHD (20%).

Ten patients having cPDp2 expansion below the median died. Four patients died from disease progression (40%), 1 from pulmonary thrombosis (10%), 1 from acute respiratory distress syndrome (10%), 1 from fungal infection (10%), 1 from viral infection (10%), and, finally, 1 from acute GvHD (10%).

DISCUSSION

The role of MSC progenitors in the setting of HSCT still needs clarifying and is still a matter of debate. Lazarus et al[21] were the first to show that it was safe to give HLA-identical MSCs to allogeneic HSCT recipients showing MSCs of donor origin in 2 of 18 patients (2% to 14% of the MSCs obtained from BM culture). A further 2 papers dealt with the role of MSCs in 2 particular HSCT settings: Bernardo et al[22] used MSCs at the time of HSCT transplantation in recipients of cord blood grafts. Although they did not notice any differences in hematological recovery or the rejection rate, there was a significant decrease of grade III and IV acute GvHD incidence in their study cohort, when compared with controls ($P = 0.05$). Ball et al[23] performed cotransplantation of MSCs, after haploidentical HSCT, showing how multiple MSC infusions were safe and effective in children with steroid-refractory aGvHD, especially when utilized early in the disease course. In addition, no increase of infections was documented.

Moreover, Kuzmina et al,[24] in a randomized prophylactic study, were able to show reduced rate of acute GVHD II-IV from 33% to 5% for patients who received MSCs, but, also, in this series, no improved survival was reported.

While all these studies agree on the safety and efficacy of MSC administration in GvHD, they do not offer any conclusive data on benefits for a better survival.[10,24] With the exception of very few studies, significant heterogeneity was observed in age, diagnoses, conditioning regimens, HLA matching, HSC source, MSC source and indication (prophylaxis or treatment), and culture conditions (Rizk et al for reviews[25]).

Some considerations can be made if our findings are compared with other studies. Our data come from a preclinical study, while others are from clinical trials, implying that the MSC growth and expansion we observed were not affected by infections, drug toxicity, etc., that might well have a role in MSC activity after transplantation. Moreover data on MSC survival after HSCT are still lacking and difficult to obtain, and very few studies have reported the engraftment of those cells, thus indicating a low engraftment of transplanted MSCs.[26–28] However, in univariate analysis, we demonstrated that the ex vivo MSC progenitor expansion is statistically associated with both reduced GVHD III-IV occurrences and lower TRM, while, as previously reported in clinical trials, no effect on OS was highlighted.

If numerous papers have reiterated the persistence of mesenchymal cells of recipient origin even after years from HSCT, a possible interpretation could be linked to a rapid effect of the transplanted MSCs within the graft that could reduce the lymphocytes' alloreactivity present in the BM. This effect would then be subsequently lost due to the entrapment of MSCs in the pulmonary rather than splenic or hepatic reticuloendothelial system. In particular, 2 recent papers dealt on the immunosuppressive ability of MSC: Klinker and colleagues were able to show a linear relation between MSC morphology and immunosuppressive activity, while Bloom and colleagues developed a reproducible assay to measure allogeneic MSC-mediated suppression of $CD4^+$ lymphocytes, showing a suppression of T-cell proliferation ranging from 27% to 88%, according to standard MSC products.[29,30]

When we focused on factors predicting a higher cPDp2 content, no significant effects for the patient or donor ages, the donor relationship, sex, type or disease, conditioning regimen, TNC dose, $CD34^+$ dose, or $CD3^+$ dose were observed, even when a subgroup analysis (related vs. unrelated donor) was carried out.

In conclusion, we found a direct relation between ex vivo MSC expansion and clinical outcomes. In univariate analysis, we have shown how the MSCs in the BM graft measured by in vitro cPDp2 are a clinical predictor of TRM and of severe acute GvHD. Despite lacking predictive patient-related, donor-related, or transplant-related factors on cPDp2 expansion, has a better prognostic value. A prospective study is ongoing to test MSC chimerism and survival following HSCT, while a larger multicenter study is warranted to confirm the role of MSC growth after HSCT.

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