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ORIGINAL ARTICLE

Engraftment kinetics and hematopoietic chimerism after reduced-intensity conditioning with fludarabine and treosulfan before allogeneic stem cell transplantation

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Abstract Reduced-intensity conditioning with fludarabine and treosulfan before allogeneic stem cell transplantation (SCT) was introduced several years ago. Although its feasibility has recently been proven, only limited data are available on myelotoxicity, engraftment kinetics, and the significance of hematopoietic chimerism using this novel conditioning regimen. To clarify these open questions, we analyzed 27 patients with various hematological diseases, who received allogeneic SCT preceded by fludarabine/treosulfan conditioning. Further assessment endpoints included graft-vs-host disease (GvHD), mortality, and overall survival (OS). Allogeneic SCT was followed by neutropenia (absolute neutrophil count $\leq 0.5 \times 10^9$/l) and thrombocytopenia (platelets $\leq 20 \times 10^9$/l) in all patients. All patients showed stable neutrophil engraftment, and all except one had stable platelet engraftment. Grades II–IV acute GvHD was found in 48% of patients, whereas 52% developed chronic GvHD. The treatment-related mortality on day +100, 1 year after SCT, and at the last follow-up was 11, 26, and 33%, respectively. We found complete chimerism rates of 46, 57, and 72% on days +28, +56, and at the last follow-up or before death, respectively. The underlying malignancy tended to relapse more frequently in patients with mixed chimerism than in those with complete chimerism on day +28 as well as on day +56 (not significant). Additionally, no significant association was found between hematopoietic chimerism and donor type, GvHD, or OS, respectively. We conclude that reduced-intensity conditioning with fludarabine and treosulfan before allogeneic SCT is myeloablative, provides stable engraftment, and leads to complete chimerism in the majority of patients.

Keywords SCT · Reduced-intensity · Fludarabine · Treosulfan · Engraftment · Chimerism

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Introduction

Allogeneic stem cell transplantation (SCT) is a potentially curative treatment for various hematological disorders. Antimalignancy effects result from cytoreduction by the pretransplantation preparative regimen and the immune-mediated graft-vs-leukemia effect [7, 22]. The latter effect plays the predominant role for a variety of hematological malignancies and was the basis for introducing reduced-intensity conditioning (RIC) regimens in the allogeneic SCT setting. This made the approach applicable for patients previously ineligible due to advanced age or comorbidity [10, 31, 33].

It has been suggested that, compared to conventional conditioning, RIC might be associated with delayed engraftment and mixed donor hematopoietic chimerism

[4]. Mixed chimerism (MC) was found to result in a higher relapse risk than complete chimerism (CC) in patients with severe aplastic anemia and chronic myeloid leukemia [16, 17]. However, the prognostic value of the degree of chimerism remains controversial in patients with acute leukemia [1, 2, 18, 35].

The nucleoside analogue fludarabine was established in RIC protocols in 1997 [4, 28, 33]. The substance is characterized by high activity against lymphoid neoplasms and has a favorable toxicity profile [19]. The alkylating agent treosulfan (L-threitol-1,4-bis-methanesulfonate; dihydroxybusulfan; NSC 39069) is a bifunctionally alkylating agent approved for a variety of solid tumors, including ovarian carcinoma and uveal melanoma [12, 30]. Recent reports have demonstrated adequate in vitro activity against acute lymphocytic leukemia and multiple myeloma cell lines of human origin [14, 24]. Because of its low non-hematological toxicity even near the maximum tolerable dose of 47 g/m^2, treosulfan offers a promising alternative to conventional conditioning agents in the autologous and allogeneic SCT setting [5, 9, 29]. First clinical experiences using this agent in the combination with fludarabine as conditioning before allogeneic SCT suggested that, despite a pronounced myelotoxicity and immunosuppressive features, the treatment-related mortality (TRM) and non-hematological toxicity are low [9].

Despite previous evidence substantiating the feasibility of conditioning with fludarabine and treosulfan, limited data are available on engraftment kinetics and the significance of hematopoietic chimerism in terms of relapse, acute graft-vs-host disease (GvHD), and survival. To clarify these open questions, we analyzed 27 patients with various hematological diseases who received allogeneic SCT preceded by fludarabine/treosulfan conditioning between 2001 and 2004.

Materials and methods

Patients and donors

Patients' characteristics are shown in Table 1. All patients were ineligible to receive conventional conditioning due to advanced age (at least 55 years; 14 patients), heavy pretreatment (previous autologous SCT or intensive conventional chemotherapy; 6 patients), previous neoplasia (2 patients), or major comorbidity (5 patients). The total group of 27 patients, all treated at the university hospital Charité—Campus Benjamin Franklin (Berlin, Germany) had a median age of 55 years (range 18–66 years) and comprised 11 women (41%) and 16 men (59%). The underlying disease was multiple myeloma (MM) in nine patients (33%), acute myeloid leukemia (AML) in eight (30%), chronic lymphocytic leukemia (CLL) in five (18%), non-Hodgkin's lymphoma (NHL) in three (11%), and Hodgkin's disease (HD) and myeloproliferative syndrome (MPS) in one patient each (4%). The stem cell source was bone marrow (BM) in five patients (19%) and peripheral blood stem cells (PBSC) in 22 (81%). Nucleated cells ($\times 10^8$/kg BW), CD34+ cells ($\times 10^6$/kg BW), and CD3+ cells ($\times 10^7$/kg BW) were administered in median amounts of 2.8 (range 1.7–3.9), 3.9 (range 2.2–6.0), and 0.7 (range 0.1–2.4), respectively, in patients receiving BM and 7.3 (range 2.5–16.9), 5.7 (range 3.9–11.2), and 21.4 (range 0.4–45.0), respectively, in those receiving PBSC. The donors were 18 human leukocyte antigen (HLA)-matched related (MRD, 67%) and 9 HLA-matched unrelated (MUD, 33%). All PBSC donors received granulocyte colony-stimulating factor (G-CSF) at doses ranging from 8 to 16 μg/kg BW (subcutaneously administered twice daily).

Conditioning therapy and GvHD prophylaxis

The conditioning regimen consisted of intravenous (IV) fludarabine (30 mg m^{-2} day^{-1}; Schering AG, Berlin, Germany) from days −6 to −2 and treosulfan (12–14 g m^{-2} day^{-1}; Medac, Wedel, Germany) from days −6 to −4. GvHD prophylaxis was carried out with cyclosporine (2×3 mg/kg BW/day IV) from day −3 onwards and short-course methotrexate (15 mg/m^2 IV on day 1, followed by 10 mg/m^2 IV on days 3 and 6). Patients transplanted from MUD additionally received ATG (Thymoglobulin®, Sangstat, Lyon, France, now Genenzym, Cambridge, MA, USA) from days −3 to −1 at a total dose of 7.5 mg/kg BW (IV). G-CSF was not administered to any of the patients after SCT. All patients received prophylaxis for infectious diseases according to the local standards.

Chimerism analyses

Chimerism analyses were based on the discrimination of donor and recipient alleles on short tandem repeats (STR) using polymerase chain reaction (PCR) with fluorescence-labeled primers. Initial genotyping to detect informative STR loci was performed using ethylenediaminetetraacetic acid (EDTA) peripheral blood (PB) from the patient and donor or graft. After transplantation, chimerism was analyzed on BM and PB samples on days +28, +56, +100, +180, +360, annually after transplantation, and as clinically indicated. In this investigation, we included chimerism data from BM without cell separation to estimate engraftment. DNA was extracted using the standard DNA extraction method (Invisorb Spin Cell Mini Kit; Invitek GmbH, Germany) as recommended by the manufacturer. The final concentration of DNA was 0.02–0.2 ng per PCR. The PCR reaction was run using the commercial GenePrint® fluorescent STR system (Promega, Madison, WI, USA).

Table 1 Patients' characteristics

No.	Diagnosis	Disease status before SCT	Hematopoietic chimerism			Acute GvHD	Relapse/disease progression	Causes of death	Survival
			Day +28	Day +56	Last follow-up				
1	MPS	Blast crisis	CC	NA	NA	IV	No	Acute GvHD	77
2	AML	PR	NA	NA	NA	0	No	Fungal sepsis	25
3	CLL	PR	MC	MC	CC	II	No	NA	1,140+
4	CLL	PR	MC	CC	CC	III	Yes	NA	456+
5	AML	1. CR	CC	CC	CC	I	No	NA	547+
6	AML	PR	MC	CC	CC	I	No	NA	853+
7	MM	PR	CC	CC	CC	III	No	NA	653+
8	MM	PD	CC	MC	MC	II	Yes	Relapse	135
9	CLL	PR	MC	MC	CC	0	No	Viral encephalitis	535
10	AML	1.CR	MC	CC	CC	II	No	NA	1,114+
11	AML	PR	MC	CC	MC	III	Yes	Myocardial infarction	101
12	AML	2. CR	CC	CC	CC	0	No	Viral encephalitis	90
13	MM	CR	CC	MC	CC	II	No	NA	960+
14	MM	PR	MC	CC	CC	II	Yes	NA	1,029+
15	NHL	PR	CC	CC	CC	I	No	NA	903+
16	MM	PD	MC	MC	MC	III	Yes	Relapse	445
17	MM	CR	MC	MC	MC	III	Yes	NA	896+
18	MM	CR	ND	ND	CC	0	No	NA	1,182+
19	CLL	PR	MC	MC	MC	0	Yes	Relapse	875
20	NHL	3.CR	CC	MC	CC	0	Yes	NA	985+
21	MM	PR	ND	ND	CC	I	Yes	NA	1,311+
22	HL	PD	MC	CC	CC	I	No	Fungal pneumonia	254
23	CLL	CR	MC	MC	MC	0	Yes	Viral encephalitis	462
24	AML	2. CR	MC	MC	CC	0	Yes	NA	896+
25	AML	2. CR	CC	CC	MC	IV	No	Chronic GvHD	143
26	NHL	1. CR	CC	CC	CC	I	Yes	Bacterial sepsis	469
27	MM	PR	CC	CC	CC	II	Yes	Relapse	420

CR Complete response, *PR* partial remission, *PD* progressive disease, *CC* complete hematopoietic chimerism, *MC* mixed chimerism, (*plus sign*) patient is still alive, *NA* not applicable, *ND* not done; for abbreviations of the diagnoses, see "Materials and methods"

For automated fluorescence fragment analysis, PCR products were denatured for 4 min at 95°C before separation in a 6% polyacrylamide denaturing sequencing gel. Electrophoresis was performed on the DNA sequencer (A.L.F™ DNA Sequencer; Pharmacia LKB Biotechnology, Uppsala, Sweden). Donor chimerism was quantified by using the fragment manager software VI.2 (Pharmacia LKB Biotechnology, Germany), which enables an easy definition, labeling of the informative STRs, and calculation of peak areas. Donor chimerism was established by calculating the mean value of all informative STR markers. The sensitivity of the method depended on the marker combinations and varied from 1 to 5% (3% median). The high resolution of the combination of eight STR loci ensured unequivocal identification of the recipient and donor in all cases.

Definitions and statistics

Myeloid engraftment was defined as the first of three consecutive days with an absolute neutrophil count (ANC) above 0.5×10^9/l. Platelet engraftment was defined as the first of seven consecutive days with a platelet count exceeding 20×10^9/l without platelet transfusion. Acute GvHD was graded according to the revised Glucksberg scale [27]. Chronic GvHD was assessed in patients alive after day +100 and scored using the revised Seattle criteria

[21]. The disease remission status and response were classified as previously published [6, 8, 11, 13]. TRM was defined as any death after allogeneic SCT not caused by relapse or disease progression. Time to progression (TTP) was defined as the time from SCT to a more advanced stage of the disease or disease progression after transient remission. Progression-free survival (PFS) was considered to be the time from SCT to death or disease progression/relapse. Overall survival (OS) was defined as the time from SCT to death or the last follow-up. CC was present, if 100% of hematopoietic cells in BM or PB were found to be of donor origin.

Analyses were performed on July 31, 2005, with a median follow-up of 547 days for the total patient population (range 25–1,311 days) using the software SPSS 12.0 for Windows XP. The donor type (MRD or MUD, respectively) was compared with the chimerism status using the exact Fischer's test. The relationship between the chimerism status and the relapse/survival rate was assessed by the log-rank test, whereas the Mann–Whitney *U* test was used to compare chimerism and GvHD. *p* values below 0.05 were considered significant (two sided). OS, TTP, and PFS were calculated using the Kaplan–Meier method. Quoted confidence intervals (CI) refer to 95% boundaries.

Results

Myelotoxicity and engraftment

Conditioning followed by SCT induced neutropenia (ANC $\leq 0.5\times10^9$/l) and thrombocytopenia (platelet count $\leq 20\times10^9$/l) in all patients. Mean myeloid engraftment was on day +15 (range day 9–24) for patients receiving PBSC and on day +16 (range day 13–20) for those receiving BM, whereas the mean platelet engraftment was on day 18 (range day 10–36). None of the patients showed primary myeloid graft failure, whereas one patient (4%) demonstrated lack of platelet engraftment up to day +167. None of the patients suffered secondary graft rejection.

Graft-vs-host disease (GvHD)

No GvHD was found in 8 patients (30%), overall grade I in 6 (22%), and grades II–IV in 13 (48%; grade II in 6 patients, grade III in 5, and grade IV in 2; Table 1). Chronic GvHD was evaluable in 23 patients. In four patients, chronic GvHD could not be assessed due to early death. Eleven patients (48%) showed no chronic GvHD, whereas limited and extensive chronic GvHD was present in seven (30%) and five (22%) patients, respectively.

Relapses and mortality

All but two patients who died early (nos. 1 and 2, Table 1) were included in the response analysis. Before allogeneic SCT, a complete response (CR) and a partial remission (PR) were achieved by 44% (11 of 25 patients) each, whereas 12% (3 of 25 patients) showed progressive disease. Allogeneic SCT resulted in ongoing remission at the last follow-up or before death in 12 (48%) of these patients (CR in 11 patients and PR in one patient). Thirteen patients (52%) suffered from relapse/disease progression of the underlying malignancy; however, part of them achieved another remission after initiation of donor lymphocytic infusion (five patients), withdrawal of immunosuppressives, second allogeneic SCT (two patients), or subsequent antimalignancy treatment. The median TTP was 740 days (CI 319–1,161 days), whereas the median PFS was 316 days (CI 0–735 days; Fig. 1a,b). The estimated PFS 1 and 2 years after SCT was 48 and 36%, respectively.

The TRM on day +100, 1 year after SCT, and at last follow-up was 11, 26, and 33%, respectively. TRM encompassed infectious complications, GvHD, and myo-

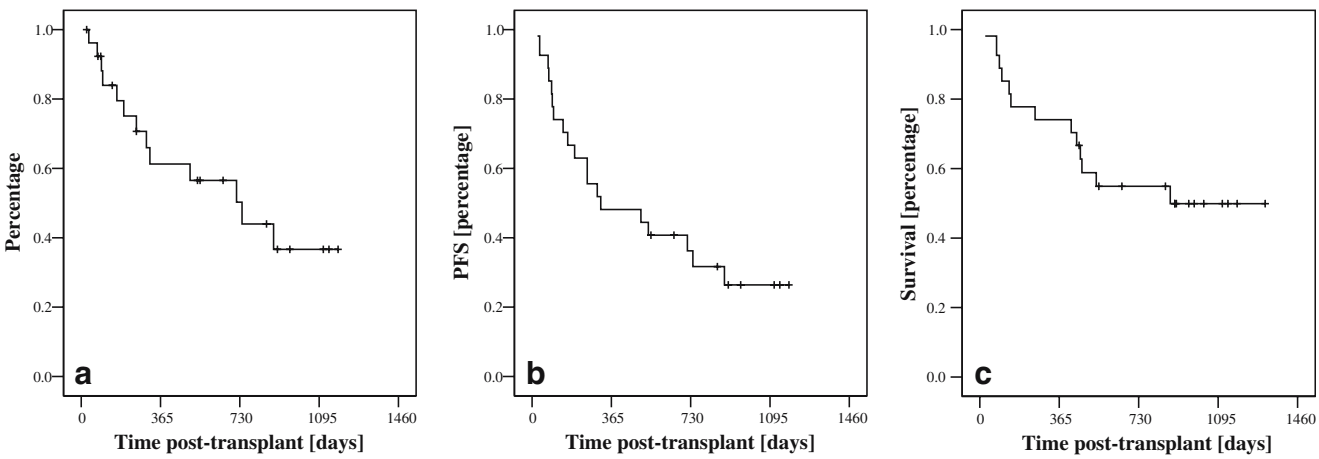

Fig. 1 Kaplan–Meier estimate of TTP (**a**), PFS (**b**), and OS (**c**)

cardial infarction in one patient (Table 1). Up to the last follow-up, four patients (15%) have died due to relapse. The median OS was 875 days; the estimated OS according to Kaplan–Meier, 1 and 2 years after SCT was 74 and 55%, respectively (Fig. 1c).

Chimerism analyses

We found CC rates of 46, 57, and 72% on days +28, +56, and at the last follow-up or before death, respectively. Chimerism analyses between day +56 and the last follow-up did not reveal any switch of chimerism status (*data not shown*). To determine chimerism changes, we defined six courses of chimerism and grouped patients accordingly (Table 2): The majority of patients either had sustained CC or switched to CC after initial MC. Noteworthy is the fact that 62% (8 of 13 patients) with initial MC had CC at the last follow-up, which occurred either spontaneously or after withdrawal of immunosuppressives.

Additionally, we investigated the association of day-28 chimerism with the donor type (MRD or MUD, respectively), acute GvHD, and OS, respectively. The association of hematopoietic chimerism with relapse was calculated on day +28 as well as on day +56. Considering the patients with CC on day +28, 56% had an MRD and 44% an MUD, whereas, in the patient cohort with MC, 69% had an MRD and 31% an MUD (p=0.81, not significant). Acute grades II–IV GvHD was comparable in patients with CC and MC on day +28 (44 and 56%, respectively; p=0.763, not significant). Analyses of the association of chimerism degree with OS and relapse encompassed 23 patients, 4 patients were excluded from this analysis due to early death (2 patients, Table 1) or missing early determination of the chimerism status (2 patients). For patients with CC on day +28, the median survival was 420 days (CI 0–973 days) and 875 days (CI not applicable) for those with MC on day +28 (p=0.33, not significant).

Relapses occurred in 33% (4 of 12 patients) with CC on day +28 but in 62% (8 of 13 patients) with MC on day +28 (p=0.44, not significant). On day +56, 5 of the 13 patients (39%) with CC relapsed during follow-up, whereas this was the case in 70% (7 of 10 patients) with MC (p=0.32, not significant).

Table 2 Course of chimerism status, two patients with early death (nos. 1 and 2, Table 1) and two with missing early chimerism analysis (nos. 18 and 21) are not included

Course of chimerism	Number of patients (%), N=23
Sustained CC	6 (26)
CC after initial MC	8 (35)
Sustained MC	4 (17)
Transient MC	2 (9)
Transient CC	1 (4)
MC after initial CC	2 (9)

CC Complete chimerism, *MC* mixed chimerism

Discussion

Conditioning with fludarabine and treosulfan followed by allogeneic SCT induced neutropenia and thrombocytopenia in all patients. All our patients demonstrated stable neutrophil engraftment, and all except one (96%) had stable platelet engraftment. Secondary graft failure was not observed in any of the patients. In contrast to this observation, a variety of fludarabine-based conditioning regimens were found to be non-myeloablative and to be associated with delayed engraftment and an increased rate of secondary graft rejection [4, 25, 34]. Our results indicate that the conditioning agent treosulfan, which has been introduced in the allogeneic SCT setting in the combination with fludarabine most recently, possesses a pronounced stem cell toxicity as well as immunosuppressive features. This hypothesis is supported by recently published in vitro and rodent model data as well first clinical experiences investigating this novel conditioning regimen [9, 26, 32, 36].

We found CC rates of 46, 57, and 72% on days +28, +56, and at the last follow-up or before death, respectively. The CC rate of 46% on day +28 after allogeneic SCT seems to be lower than that 80–90% usually achievable by conventional conditioning early after SCT. However, 62% of patients with initial MC converted to CC during follow-up, which occurred either spontaneously or withdrawal of immunosupressives. Opposed to this observation, various RIC regimens were found to be associated with persistence of MC [4].

Beside the course of hematopoietic chimerism, we also investigated its association with donor type, GvHD, OS, and relapse, respectively.

In our analysis, patients with MC on days +28 and +56 tended to have a higher risk of relapse than those with CC (62 and 70% vs 33 and 39%, respectively), although statistical significance was not reached. Recently, Lamba et al. [20] found that day +30 chimerism failed to provide any prognostic information, although MC on day +90 did correlate with an increased relapse risk. Our data indicate that early hematopoietic chimerism might be informative in terms of the relapse.

Interestingly, patients with MC on day +28 tended to have a longer OS than those with CC. This observation might be partly explained by the fact that some of the patients with relapse or disease progression after SCT showed again a remission after initiation of immunotherapeutic strategies or subsequent antimalignancy therapy. Thus, considering the limited follow-up in our analysis,

the higher relapse rate of patients with MC compared to those with CC does not seem to shorten their OS. As it has been shown previously that MRD- and MUD-transplantation are associated with a comparable OS [37], we do not assume that the slightly higher percentage of MUD than MRD among patients with CC compared to those with MC explain the difference in OS.

We found that the acute GvHD rates did not differ significantly between patients with CC and those with MC. CC was previously thought to be associated with a higher rate of acute and chronic GvHD than MC due to higher "alloreactivity" in patients with CC [3, 16, 23]. As we performed chimerism analyses in unfractionated cells, we cannot exclude the possibility that donor chimerism of unfractionated cells may not reflect the chimerism of T cells, which predominantly mediate GvHD [2, 15].

The 48, 52, and 15% rates of grades II–IV acute GvHD, chronic GvHD, and death due to relapse observed in our investigation are comparable to the 41, 39, and 22% rates described in a meta-analysis including patients who have undergone RIC or non-myeloablative conditioning [4]. Considering the extensive pretreatment of our patient cohort, the median OS, TTP, and PFS of 875, 740, and 316 days, respectively, seems to be promising.

We conclude that RIC with fludarabine and treosulfan before allogeneic SCT possesses sufficient stem cell toxicity and immunosuppressive features to provide stable engraftment, low rate of secondary graft rejection, and CC in the majority of patients. Patients with MC on day +28 as well as on day +56 tended to relapse more frequently than those with CC; however, statistical significance was not reached.

Surely, our patient cohort is heterogeneous with respect to donor type, stem cell source, GvHD prophylaxis, use of ATG, and some other aspects. As these factors are well known to influence the risk of relapse and occurrence of GvHD, prospective trials with less heterogeneity are warranted to confirm our findings.

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