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Content of Endothelial Progenitor Cells in Autologous Stem Cell Grafts Predict Survival after Transplantation for Multiple Myeloma

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A B S T R A C T

Multiple myeloma (MM) is considered an incurable B cell malignancy, although many patients can benefit from high-dose therapy with autologous stem cell transplantation (ASCT) as a first-line treatment. In non-Hodgkin lymphoma (NHL), ASCT is usually performed after relapse with curative intent. Disease progression is often associated with increased angiogenesis, in which endothelial progenitor cells (EPC) may have a central role. Here, we investigated the clinical impact of EPC levels in peripheral blood stem cell (PBSC) autografts for MM and NHL patients who received ASCT. EPC were identified by flow cytometry as aldehyde dehydrogenase^{hi} CD34^{+} vascular endothelial growth factor receptor 2^{+} CD133^{+} cells in both MM and NHL autografts. In MM, there was a positive correlation between EPC percentage and serum (s)-β_2-microglobulin levels ($r^2 = .371$, $P = .002$). Unlike for NHL patients, MM patients with high numbers of infused EPC (EPC cells per kilogram) during ASCT had significant shorter progression-free survival (PFS) ($P = .035$), overall survival ($P = .044$) and time to next treatment ($P = .009$). In multivariate analysis, EPC cells per kilogram was a significant independent negative prognostic indicator of PFS ($P = .03$). In conclusion, the presence of high number of EPC in PBSC grafts is associated with adverse prognosis after ASCT in MM.

INTRODUCTION

Multiple myeloma (MM) is a malignant disorder characterized by clonal expansion of postgerminal-center malignant B cells in the bone marrow [1-3]. High-dose chemotherapy followed by autologous stem cell transplantation (ASCT) is considered the standard first-line therapy for patients <65 years of age [4]. Survival ranges from a few months to more than 20 years, and several prognostic indicators have been established. Median progression-free survival (PFS) for patients who achieve a complete response (CR) after ASCT is significantly longer compared with non-CR patients [5]. Moreover, high-risk patients with t(4;14) or del(17p) have a poor prognosis after ASCT [6-9]. These patients may actually achieve CR, although at a lower rate, but early relapses are more common [6]. For stratification of MM patients at time of diagnosis, the International Staging System (ISS) is a simple and reliable tool that includes β_2-microglobulin and albumin [10]. Prognostic indicators and biomarkers are useful and have additive value when they also give insight into biological mechanisms.

Disease progression in MM is accompanied by an increase of bone marrow angiogenesis [11,12]. High level of vascular endothelial growth factor (VEGF) levels in peripheral blood from MM patients has been reported to be associated with more advanced disease, and levels of VEGF in bone marrow specimens correlate with β2-microglobulin levels [13]. Myeloma cells have no or only weak expression of VEGF receptor (VEGFR) 1 and 2. However, VEGF-A stimulation of

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stromal and microvascular endothelial cells has been shown to increase secretion of IL-6, a potent growth and survival factor for myeloma cells [14]. Accordingly, high levels of IL-6 are associated with adverse prognosis in MM [15].

Endothelial progenitor cells (EPC) were first characterized by Asahara in 1997 based on coexpression of the surface markers VEGFR2 and CD34 [16]. Later studies have confirmed that EPC express CD34 [17,18], VEGFR2 [18-20], and also CD133 [18,19,21]. Primitive hematopoietic progenitor cells from bone marrow and umbilical cord blood express high levels of cytoplasmic aldehyde dehydrogenase (ALDH) as compared to lymphocytes and monocytes [22]. Furthermore, a fluorescent substrate of ALDH (Aldefluor, Stem Cell Technologies, Manchester, United Kingdom) can be used to identify cells with increased ALDH activity [23]. Hence, an interesting strategy would be to identify EPC according to a conserved stem cell function ($ALDH^{hi}$) combined with phenotypic markers.

Based on previous studies documenting the importance of angiogenesis in MM, we hypothesized that levels of EPC in stem cell grafts would be associated with clinical outcome after ASCT. The aim of the present study was to explore this by investigating the presence of $ALDH^{hi}CD34^{+}VEGFR2^{+}CD133^{+}$ EPC by flow cytometry technology in autologous peripheral blood stem cell (PBSC) grafts from patients with MM and from patients with non-Hodgkin lymphoma (NHL) as a comparison.

MATERIAL AND METHODS

Patients

Forty-one patients (MM; n = 24, NHL; n = 17) with available cryopreserved PBSC autograft samples collected in the period between 1995 and 2006 were included in this study. MM patients received induction therapy with either vincristine 1.6 mg/m^2, doxorubicin 36 mg/m^2, and dexamethasone 40 mg (VAD) or cyclophosphamide 1000 mg/m^2 and dexamethasone 40 mg (Cy-Dex) as previously described [24]. PBSC harvest was performed after 1 cycle of cyclophosphamide (2 g/m^2) followed by filgrastim. MM patients received melphalan (200 mg/m^2) conditioning before transplantation [24]. NHL induction therapy and mobilization of PBSC are described in Supplemental Materials. The study was approved by Regional Committee for Medical Research Ethics (REK-Nord 2011/724).

PBSC Collection and Cryopreservation

PBSC were collected on a Cobe Spectra Apheresis Instrument (Cobe Laboratories, Gloucester, UK). Cells were subsequently treated to a concentration of 100 to 200 $\times$ 10^6/mL and mixed with dimethyl sulfoxide (DMSO) to a final concentration of 10% DMSO before freezing in the gas phase of liquid nitrogen. Small aliquots of 1 mL PBSC from all patients were used in this study.

Reagents and Antibodies

Quantification of EPC

Human IgG, reagent grade I4506 was from Sigma-Aldrich (Saint Louis, MO). Aldefluor was from StemCell Technologies. Antihuman VEGFR2-PE (clone 89106) was from R&D (Abingdon, United Kingdom). Antihuman CD34-PE-Cy7 (clone 8G12) was from BD Biosciences (San Jose, CA), antihuman CD133-APC (clone AC133) was from Miltenyi Biotec (Lund, Sweden).

Viability analysis

Antihuman CD34-PE (clone 8G12) and via-probe (7AAD) was from BD Biosciences and antihuman CD45 FITC (clone T29/33) was from Dako (Glostrup, Denmark).

Quantification of clonal circulating plasma cells

Antihuman CD19-PE-Cy7 (clone J3-119) and antihuman CD38-APC Alexa750 (clone LS198-4-3) was from Beckman Coulter (Brea, CA). Antihuman CD20-Horizon V-450 (clone L27) and antihuman CD45-Horizon V-500 (clone 2D1) was from BD Biosciences. Antihuman CD138-APC (clone MI15), Kappa Light Chains-FITC, and Lambda Light Chains-PE (code number FR481) were from Dako. CellFIX (catalog number 340181) was from BD Biosciences. Permeabilization Medium (catalog number GAS002S-100) was from Life Technology (Thermo Fisher Scientific, MA).

Analysis of EPC and Clonal Circulating Plasma Cells in Stem Cell Grafts by Flow Cytometry

Cryopreserved PBSC were thawed, washed in PBS with .2% bovine serum albumin (PBSA), and counted. To block Fc receptor binding, 5 $\times$ 10^6 cells were incubated with 5 μg human IgG in 15 minutes at 4°C. Cells were then washed, 400 μL Aldefluor assay buffer was added, and cells were incubated with 5 μL/.61μg Aldefluor for 30 minutes at 37°C. Diethylaminobenzaldehyde, a specific ALDH inhibitor, was used as a negative control, as previously described [25]. Cells were then washed, and 200 μl Aldefluor Assay Buffer was added. Then, cells were costained with 10 μL anti-VEGFR2-PE, 2.5 μL anti-CD34-PE-Cy7, and 10 μL anti-CD133-APC for 30 minutes at 4°C. In a separate tube, 3 $\times$ 10^6 cells in .2% PBSA were incubated with 5 μL anti-CD45-FITC, 10 μL anti-CD34-PE, and 20 μL 7AAD. The cells were then washed, resuspended in Aldefluor assay buffer or PBS, respectively, and stored on ice protected from light until they were collected on a FACSCanto flow cytometer (Becton Dickinson, Franklin Lakes, NJ). For quantification of clonal circulating plasma cells (cPC), 5 $\times$ 10^6 cells were incubated with 2.5 μL CD19-PE-Cy7, 2.5 μL CD20-Horizon V450, 5 μL CD38 APC-Alexa 700, 2.5 μL CD45-Horizon V500, and 10 μL CD138-APC for 20 minutes, dark in room temperature. The cells were then fixated, washed, and resuspended before incubated with 10 μL kappa/lambda-FITC/PE and 100 μL permeabilization medium for 15 minutes, dark, in room temperature. The cells were washed, resuspended in PBSA, and collected on a FACSCanto II flow cytometer (Becton Dickinson). Flow cytometry data were analyzed using FlowJo v7.6.5 (TreeStar, Inc., Ashland, OR).

Statistics, Definitions, and Endpoints

GraphPad Software (La Jolla, CA) was used to determine statistical significance of difference between groups by applying unpaired *t*-test or Mann-Whitney test as described in figure legends. Survival curves were plotted using Kaplan-Meyer method and comparisons were based on log-rank test with a significance level of $P < .05$. For multivariate analyses, a Cox proportional hazards model was performed with SPSS version 21 (IBM Corporation, NY). *EPC percentage* was defined as percentage of $VEGFR2^{+}CD133^{+}$ cells in the $CD34^{+}$ population. *EPC cells per kilogram* was defined as a ratio of EPC (percent of $CD34^{+}$ population) as determined by flow cytometry measurements, divided by number of stem cells infused during ASCT ($CD34^{+}$ cells $\times$ 10^6/kg). PFS was measured from PBSC collection to date of progression or death. Patients who had not progressed or relapsed were censored on the last date they were known to be alive. Overall survival (OS) was calculated from PBSC collection to date of death or last visit. *Time to next treatment* (TNT) was defined as the time from collection of PBSC to the onset of new chemotherapy or radiation therapy after ASCT [26]. Disease progression was defined according to International Myeloma Working Group Response Criteria [27]. Data on immunofixation was not available. Hence, *near CR* was defined as absence of detectable monoclonal component in the blood and urine electrophoresis and <5% plasma cells in bone marrow. *Very good partial response* was defined as a 90% or more decrease in the serum monoclonal component level (or urine monoclonal component lower than 100 mg/24 hours in Bence-Jones MM). *Partial response* was defined as a 50% to 89% decrease in the serum monoclonal component level or a 90% or more decrease in urine monoclonal component [28,29].

RESULTS

Patient Characteristics

In this study, we included PBSC autograft samples from 24 MM patients and 17 NHL patients. The median age for the MM cohort at ASCT was 55.3 years, and median observation time after ASCT was 10.2 years. The MM patients' characteristics at onset of therapy are presented in more detail in Table 1. The NHL patients' characteristics at onset of therapy are summarized in Supplemental Table S1 and Supplemental Materials.

Identification of $CD34^{+}VEGFR2^{+}CD133^{+}$ EPC Population with High ALDH Activity in PBSC Grafts from NHL and MM Patients

We aimed to characterize the frequencies of EPC in PBSC autograft samples from NHL and MM patients by stem or progenitor cell properties as determined by high activity of intracellular ALDH, combined with surface expression of CD34, VEGFR2, and CD133. The gating strategy is outlined in Figure 1A. The cells with high ALDH activity accounted for an average 4.33% and 3.06% in NHL and MM patient samples,

respectively. Approximately 90% of the cells within the ALDH^{hi} population were CD34^{+} (Figure 1A). Furthermore, back-gating analysis showed that the majority of VEGFR2^{+}CD133^{+} in the autografts also were CD34^{+}ALDH^{hi} (Figure 1B).

The median percentage of 7AAD^{neg} cells within an intact cell gate was 83.2%, thus showing that the quality of the patient samples was good. For all samples, only cells within the live cell gate were included in the analysis. Within this live cell gate, consisting of lymphocytes and monocytes, the median percentage of CD45^{+}CD34^{+}7AAD^{neg} cells was 93.5% and, hence, limiting the issue of dead cells confounding the results.

We found that CD34^{+}VEGFR2^{+}CD133^{+}ALDH^{hi} EPC were present in stem cell grafts from both NHL and MM patients, but at highly variable frequencies, ranging from .02% to 7.56% of CD34^{+} cells (Figure 1C). When comparing NHL and MM, our analysis did not reveal any significant differences in the percentage ALDH^{hi} cells, CD34^{+} cells $\times$ 10^{6}/kg, EPC percentage, or EPC cells per kilogram (data not shown). MM patients had no difference in OS, PFS, or TNT according to the induction chemotherapy (VAD versus Cy-Dex). Furthermore, no significant difference in the EPC percentage within the MM cohort according to induction chemotherapy before ASCT (VAD versus Cy-Dex) was found (data not shown). Thus, variations in percentage of EPCs could not be explained by diagnosis or type of chemotherapy treatment in this cohort.

Number of EPC Reinfused during ASCT Predicted Adverse Outcome in MM Patients

We observed that both MM and NHL patients had highly variable frequencies of EPC in PBSC grafts (Figure 1C) and we went on to analyze if levels of EPC were associated with clinical outcomes. Clinical and flow cytometry data from MM patients with percentage of EPC higher or lower than the cohort median are presented in Table 2. Survival analysis showed that MM patients with EPC percentage higher than cohort median had significant shorter TNT ($P = .023$) but not PFS or OS (Supplemental Figure 1A). In contrast, no trend towards adverse clinical outcome for NHL patients with high EPC percentage in PBSC grafts was observed (not shown).

We then hypothesized that the actual amount of EPC per kilogram infused during ASCT, termed *EPC cells per kilogram*, might be an even stronger predictor for outcome than percentage EPC in the MM cohort. EPC cells per kilogram ranged from .02 to 2.37, with a median of .24 (Figure 2A). Survival analysis showed that MM patients with higher than cohort median EPC cells per kilogram had shorter PFS ($P = .035$) (Figure 2B) and OS ($P = .044$) (Figure 2C), and also significant shorter TNT ($P = .009$) (Supplemental Figure 1B).

In MM, EPC cells per kilogram was a significant independent negative prognostic indicator for PFS by multivariate analyses (hazard ratio, 3.44; $P = .03$) (Table 3). Only variables with significant P values from univariate analyses were entered into the multivariate analysis, using the Cox proportional hazards model (backward stepwise, probability for stepwise entry and removal was set at .05 and .10). P values $< .05$ were considered statistically significant.

In conclusion, a high number of EPC infused (EPC cells per kilogram) during ASCT was found to be a negative prognostic factor for PFS, OS, and TNT in MM patients.

EPC Level in Stem Cell Grafts was Associated with Increased Pre-treatment s-β_2-microglobulin but not ISS Score in the MM Cohort

We found a significant positive correlation between EPC percentage in PBSC grafts and the level of s-β_2-microglobulin at baseline (Figure 3) ($r^2 = .371$, $P = .002$). In contrast, there were no associations between EPC percentage and the levels of s-albumin or s-LD (elevated versus normal) (data not shown). We found no associations between high numbers of EPC and the percentage of plasma cells in bone marrow at time of diagnosis or before ASCT, $r^2 = .0097$ and $r^2 = .0121$, respectively. MM patients with ISS I (n = 12) had significant longer OS but not PFS after ASCT compared with MM patients

Table 1
MM Patient Characteristics at Onset of Therapy

ID	Sex	Age	PC, %	s-IgA, g/L	s-IgG, g/L	s-β2-M, mg/L	s-Alb, g/L	s-LD Above Normal	s-Hb, g/dL	Initial Therapy	cPC%
3	M	59	41	.2	42.4	9.80	35.8	-	13.4	Cy-Dex	.001953
4	M	56	10	.9	41.8	1.60	36.2	No	11.6	Cy-Dex	.000015
5	M	52	23	32.5	1.7	2.40	45.8	No	11.5	Cy-Dex	.000415
6	M	56	34	.9	38.6	1.50	33.4	No	12.5	Cy-Dex	.000083
7	F	53	71	33.5	4.3	17.60	30.2	Yes	9.7	Cy-Dex	.000049
8	M	60	13	.5	3.1	3.40	42.1	No	11.7	VAD	.000779
10	M	50	46	.1	85.5	5.30	34.6	Yes	8.1	VAD	.000236
12	F	63	19	.2	21.9	2.00	40.7	No	10.8	VAD	.000050
17	F	58	20	.6	9.1	7.28	46.2	Yes	9.1	VAD	.000063
20	F	65	22	.2	85.6	4.97	29.3	Yes	9.2	VAD	.000022
23	F	48	38	.2	48.6	3.39	31	-	10	Cy-Dex	.002392
25	F	59	-	.8	10.3	1.47	42.1	No	13.1	VAD	.000069
26	F	54	68	33.7	3.0	1.95	41.4	Yes	7.3	VAD	.000275
28	M	60	15	.4	28.7	1.19	41.6	Yes	9.9	VAD	.000631
32	M	41	20	.7	69.8	2.61	35.3	No	10	VAD	.000122
36	F	57	28	.1	105.0	-	22.8	No	8.2	VAD	.000791
41	M	54	38	.9	9.4	9.31	45.1	No	12.1	VAD	.000030
42	M	56	7	.4	5.3	1.85	47.7	No	12.2	VAD	.000218
46	M	49	70	.0	1.7	1.98	44.8	No	13.5	VAD	.005969
47	M	50	70	.4	106.7	3.84	22.5	No	9.1	VAD	.000236
48	M	55	1	1.0	13.3	9.60	33.7	Yes	8.8	VAD	.000158
49	M	53	72	.1	72.1	5.06	29	No	9.7	VAD	.000094
52	F	49	1	1.2	9.4	1.20	39.8	-	10.1	VAD	.000080
54	M	56	20	15.4	6.0	1.50	40.2	No	12.2	VAD	.000043

ID indicates patient identity number; PC, plasma cells in bone marrow; s-β2-M, serum (s)-β2-microglobulin; s-Alb, s-albumin; LD, s-lactate dehydrogenase; s-Hb, s-hemoglobin; M, male; F, female.

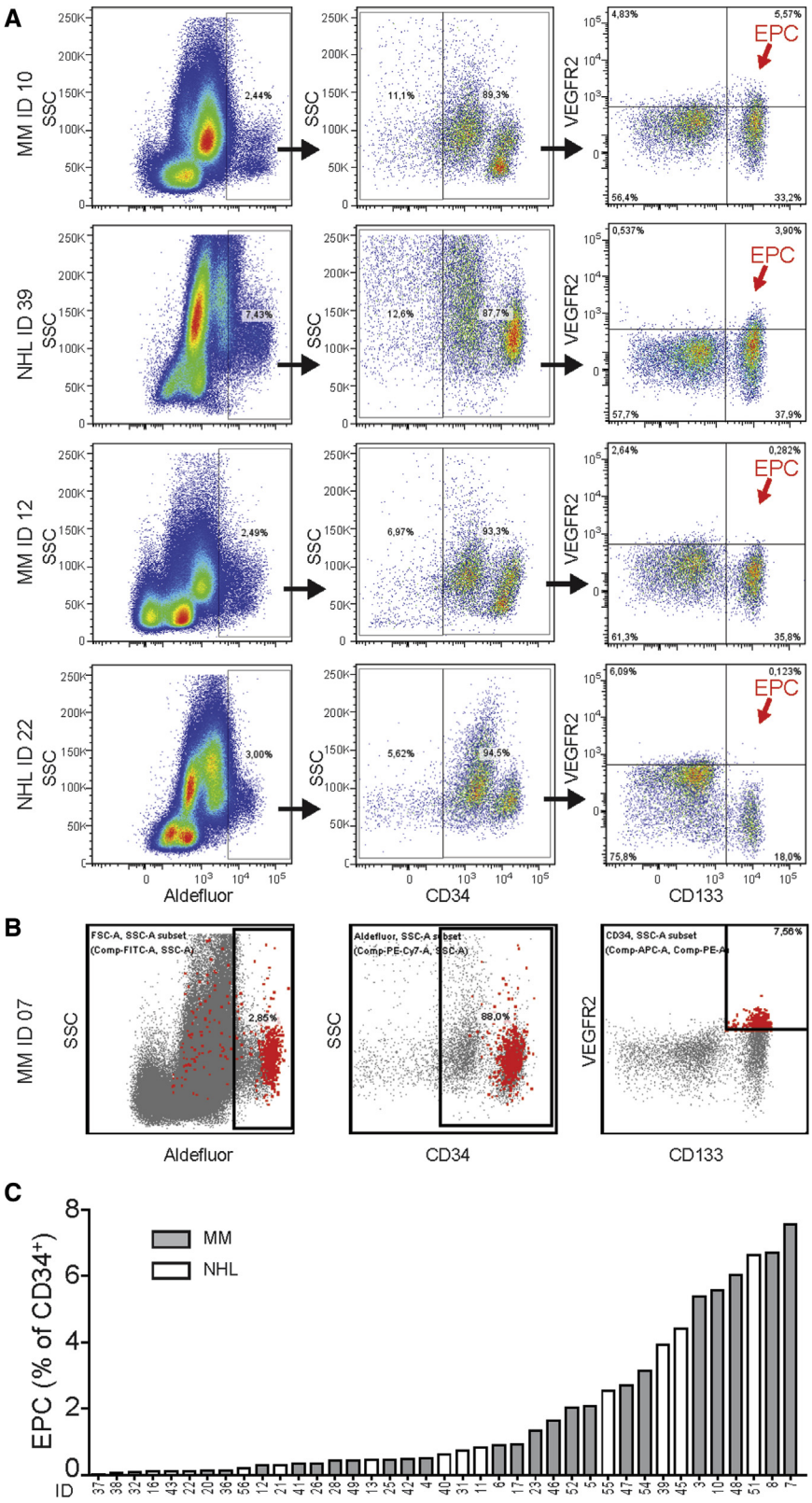

Figure 1. $CD34^{+}CD133^{+}VEGFR2^{+}$ EPC can be identified within $ALDH^{hi}$ population in stem cell grafts from NHL and MM patients. (A) Flow cytometry analysis of EPC in representative stem cell grafts from NHL and MM patients (ID 10, 39, 12, and 22). FSC/SSC gating was used to identify lymphocytes and monocytes, followed by gating on $ALDH^{hi}$ cells in order to define cells with stem or progenitor characteristics. EPC were subsequent defined as triple positive $CD34^{+}VEGFR2^{+}CD133^{+}$ cells (red arrow). (B) Back-gating analysis of VEGFR2+CD133+cells shows that the majority of double positive $VEGFR2^{+}CD133^{+}$ fall within CD34 and ALDH gates. Representative sample from a MM patient (MM ID 07). (C) Bar chart illustrating distribution of EPC as percentage of $CD34^{+}$ cells in stem cell grafts from patients treated with ASCT. NHL (white bars) and MM (grey bars).

Table 2
Clinical and Flow Cytometry Data from MM Patients with Percent EPC Higher or Lower than Cohort Median

	EPC Low Group (mean)	EPC High Group (mean)	Unpaired *t*-Test *P* Value
Age	56.09	53.21	.18
PC (%)	27.75	34.50	.68
s-β2-M (mg/L)	2.79	5.61	.09
s-Alb (g/L)	37.05	37.23	.95
s-Hb (g/L)	10.55	10.60	.95
SR (mm/h)	50.67	68.75	.22
MFI CD133	2637	3718	.10
MFI VEGFR2	140.5	213.3	.09
CD34 × 10^6/kg	4.15	5.46	.26

SR indicates erythrocyte sedimentation rate; MFI, median fluorescence intensity.

ISS II and III (n = 11) ($P = .019$) (Figure 4A,B). However, we found no differences in EPC percentage and EPC cells per kilogram between the ISS I and II and III subgroups (Supplemental Figure 2A,B). In summary, the percentage of EPC in stem cell grafts was correlated with s-β_2-microglubulin levels at baseline in the MM cohort but not with other relevant clinical prognostic parameters.

We also enumerated the number of cPC in the graft to investigate a potential correlation between EPC and cPC. Gating strategy is outlined in Supplemental Figure 3A. cPC percentage indicates clonal cells as a percentage of cells within a live lymphocyte-monocyte gate. We found highly variable frequency of cPC in the MM graft, ranging from .000015% to .005969% of live cells within lymphocyte-monocyte gate (Table 1). There were no significant correlation between EPC and cPC ($P = .82$, $r^2 = .0023$) (Supplemental Figure 3B). MM patients with lower than cohort median of cPC had trend towards better OS, PFS, and TNT ($P = .0056$, $P = .084$, $P = .098$, respectively) compared with MM patients with higher than cohort median of cPC, although this was not statistical significant in this relatively small cohort (Supplemental Figure 4A-C). EPC was still an independent negative prognostic indicator for PFS in multivariate analysis when cPC was included (hazard ratio, 3.44; $P = .027$; 95% confidence interval, 1.149 to 10.294).

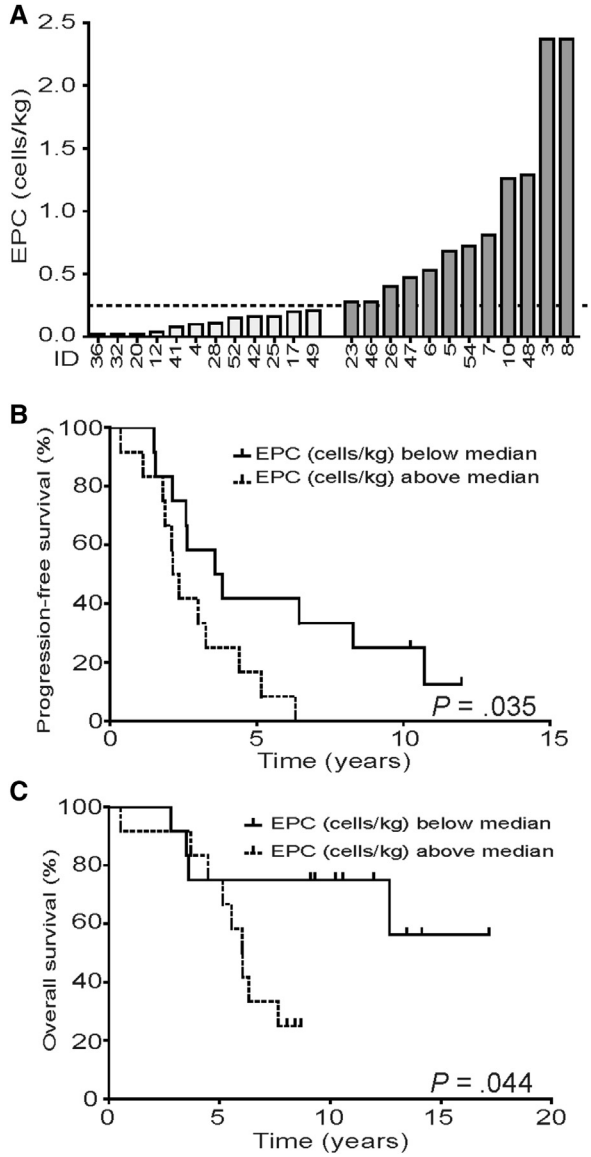

Figure 2. The absolute number of EPC in stem cell grafts stratifies OS in MM patients after high-dose chemotherapy with ASCT. (A) Bar chart illustrating the estimated number of EPC infused to MM patients together with autologous $CD34^+$ stem cells during ASCT (n = 24). EPC cells per kilogram was defined as a ratio of measured percentage EPC of $CD34^+$ cells as determined by flow cytometry analysis, divided by the total number of stem cells reinfused ($CD34^+$ cells × 10^6/kg). MM patients were divided into two groups depending on whether the number of EPC cells per kilogram was above or below the median value for the cohort. (B) Progression-free survival after ASCT in MM patients with higher or lower than cohort median EPC cells per kilogram was compared with Kaplan-Meyer plot with log-rank test and found to be significantly lower in the group with EPC cells per kilogram higher than cohort median. (C) MM patient OS after ASCT was compared with Kaplan-Meyer plot with log-rank test and found to be significant lower in MM patients with EPC cells per kilogram higher than cohort median.

DISCUSSION

Aberrant angiogenesis is one of the important hallmarks in the multistep pathogenesis of MM disease progression [30]. A central part in the complex process of malignant angiogenesis is recruitment of $VEGFR2^+$ EPC and $VEGFR1^+$ hematopoietic precursor cells from bone marrow [20]. However, the exact role of EPC in MM disease progression and clinical outcome is not yet clearly understood. In the present study, we determined the levels of EPC in PBSC autograft samples and demonstrated that MM patients with a high load of EPC in grafts had adverse PFS and OS after ASCT. Of note, EPC cells per kilogram was a significant independent negative prognostic indicator of PFS also in multivariate analysis.

We demonstrated that EPC could be detected in autologous stem cell grafts from NHL and MM patients at variable frequencies. However, we found no differences in EPC frequencies between NHL and MM patient samples, although stem cells grafts were mobilized with different protocols in the 2 cohorts. This is in line with previous work showing that there was no significant difference in EPC levels between MM and NHL after mobilization to peripheral blood by cyclophosphamide and granulocyte colony–stimulating factor [31]. Unlike for MM, we could not observe any trends towards worse outcome in NHL patients with high levels of EPC. Accordingly, the role of angiogenesis in diffuse large B cell lymphoma measured by microvessel density has shown different results in regard to clinical outcome [32,33]. MM cells grow and expand almost exclusively in the bone marrow [34], and both osteoblastic and vascular niches can support the proliferation of MM cells [35]. This emphasizes

Table 3
Results of Cox Regression Analysis Summarizing Significant Independent Prognostic Factors

Factor	Hazard Ratio	95% CI	*P* Value
EPC (cells/kg)			
Low	1		
High	3.44	1.15-10.29	.03
Induction chemotherapy			
CR/VGPR	1		
PR/SD	7.90	1.71-36.40	.008
Response after ASCT			
CR/VGPR	1		
PR/SD	3.75	1.24-11.29	.02

CI indicates confidence interval; EPC, endothelial progenitor cells; CR, complete response; VGPR, very good partial response; PR, partial response; SD, stable disease; ASCT, autologous stem cell transplantation.

bone marrow angiogenesis as an attractive target for treatment of MM. Patients with relapsed or refractory MM, including patients after ASCT, have significantly improved OS after treatment with lenalidomide [36]. Maintenance therapy with lenalidomide after ASCT increases PFS [37] and OS [38]. Lenalidomide has diverse mechanisms of action and affects angiogenesis, immune cells, and tumor cells, although the relative impact in different cell types is still unclear [39,40]. However, as VEGFR2 on MM endothelial cells can be targeted by lenalidomide resulting in impaired VEGFR2 and ERK signaling, which is involved in migration and tubular-like formation of endothelial cells [41], it would be of interest to study whether levels of EPC in PBSC grafts could predict response to lenalidomide and other antiangiogenic therapies in MM. Furthermore, it has previously been demonstrated that functional inactivation of VEGFR2 by the monoclonal antibody anti-VEGFR2 (DC101) stopped ongoing angiogenesis and tumor cell invasion [42].

Recently, a phase 3 trial with monoclonal antibody VEGFR2 antagonist demonstrated clinical efficacy with improved OS in patients with advanced gastric cancer [43]. However, the results of a phase 3 trial in breast cancer patients have been disappointing [44]. Therefore, a clinical trial investigating the incorporation of a monoclonal antibody against VEGFR2 in combination with lenalidomide as an augmented maintenance in patients with high load of EPC in stem cell grafts would be of interest.

Of importance, we found a correlation between EPC in stem cell grafts and s-β2-microglobulin. In MM, β2-microglobulin is an important prognostic factor [45-47]. The association between the levels of EPC in stem cell grafts and β2-microglobulin in peripheral blood at time of diagnosis are concordant with previous studies in MM, which have reported a correlation between β2-microglobulin and circulating endothelial cells [48] or circulating EPC [49]. The association between β2-microglobulin and EPC highlights the unsolved question whether levels of EPC in stem cell grafts has a direct effect on relapse or purely acts as a surrogate marker. The correlation between β2-microglobulin before treatment and EPC in the graft could indicate that MM patients with high tumor load at baseline mobilize more EPC together with PBSC. The presence of cPC cells has been shown to be associated with adverse outcome in MM after ASCT [50,51]. A possible mechanism for EPC mobilization could be presence of cPC. However, we found no correlation between EPC and cPC in this MM cohort. Previously, purging of stem cell graft by CD34 selection has no beneficial impact on long-term outcome in MM [52,53]. Nevertheless, actively purging of EPC in stem cell grafts would be an interesting strategy in future protocols.

In the present study, we defined EPC as progenitor cells with high intracellular ALDH expression combined with the phenotypic surface markers CD34, CD133, and VEGFR2.

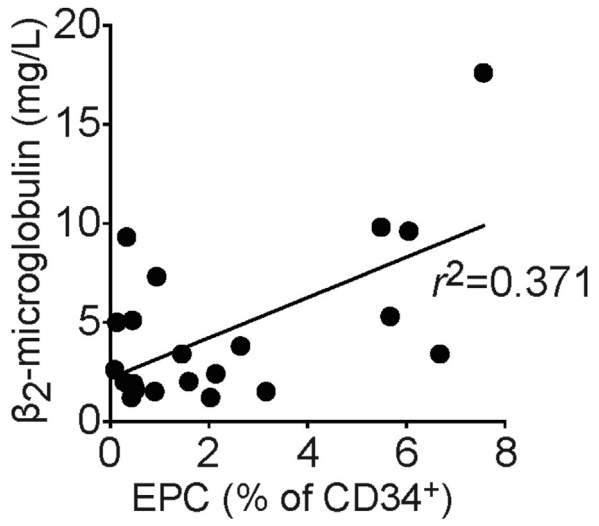

Figure 3. Positive correlation between s-β2-microglobulin and the percentage of EPC in stem cell grafts from MM patients. Scatter plot of EPC as percentage of $CD34^+$ cells in stem cell grafts as determined by flow cytometry versus s-β2-microglobulin (mg/L) in MM patients at onset of treatment (n = 23). Association between variables was evaluated by Pearson R^2.

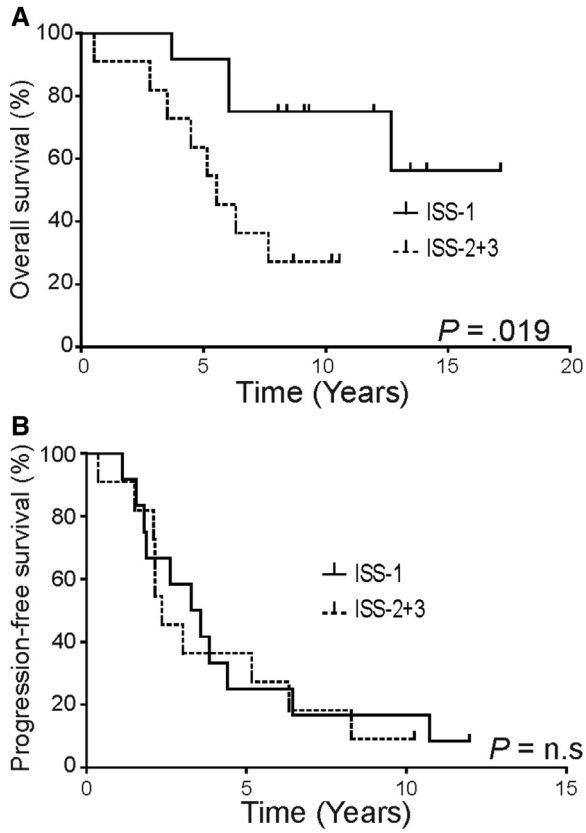

Figure 4. ISS stratifies OS in MM patients after ASCT. OS (A) and PFS (B) for patients MM patients after ASCT according to ISS I (n = 12) compared with ISS II or III (n = 11). Kaplan-Meyer plot with log-rank test. Significance level of $P < .05$.

Although the properties of EPC to differentiate into mature endothelial cells in vitro and to contribute to vessel formation after transplantation was described more than a decade ago [16], no consensus has been reached regarding a uniform definition of EPC. EPC characterized as $CD34^{+}CD133^{+}VEGFR2^{+}$ has previously been identified in NHL and MM [31], non-small cell lung cancer [54,55], myelofibrosis with myeloid metaplasia [56], and glioma [57]. However, whether $CD34^{+}VEGFR2^{+}CD133^{+}$ cells have angiogenic or hematopoietic capacities is controversial [58]. These markers are also demonstrated to be expressed on hematopoietic stem and progenitor cells, making it difficult to distinguish between endothelial and hematopoietic progenitors [59,60]. Furthermore, $ALDH^{hi}CD133^{+}$ cells have ability of multilineage reconstitution and possessed long-term repopulating ability in secondary murine recipients [61]. Therefore, high ALDH activity is a functional marker of both hematopoietic and nonhematopoietic bone marrow derived progenitor cells [62]. Recently, EPC has been characterized solely as $ALDH^{hi}$ or as $CD34^{+}CD133^{+}$ cells [63].

Although the present study included a limited number of MM patients, we found a significant correlation between increased levels of $ALDH^{hi}CD34^{+}VEGFR2^{+}CD133^{+}$ EPC in stem cell grafts and adverse clinical outcome after ASCT. Of note, the actual number of EPC infused was shown to be an independent risk factor. Although this is a retrospective study and the results have to be confirmed by prospective studies with a predefined plan for analyses in MM patient cohorts treated with novel agents such as thalidomide, lenalidomide, and bortezomib, the significant adverse outcome in a limited patient cohort indicates an evident difference caused by EPC. We conclude that further studies are warranted to confirm whether the EPCs in the stem cells grafts facilitate relapse by direct action or serve as a surrogate marker for outcome.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2014.12.027.

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