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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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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,