Leukemia Research 47 (2016) 22–25

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Increase of endothelial progenitor cells in acute graft-versus-host disease after allogeneic haematopoietic stem cell transplantation for acute myeloid leukaemia

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ARTICLE INFO

Article history:
Received 20 March 2016
Received in revised form 4 May 2016
Accepted 15 May 2016
Available online 17 May 2016

Keywords:
Acute graft-versus-host disease
Endothelial progenitor cells
Haematopoietic stem cell transplantation
Steroid-refractory

ABSTRACT

Introduction: Circulating endothelial progenitor cells (EPCs; CD31+ CD34^{bright}CD133+ CD45^{dim} cells) are novel markers of endothelial dysfunction and related to inflammatory processes such as acute graft-versus-host disease (aGvHD).
Patients and methods: 47 patients with acute myeloid leukaemia (AML) who were in complete remission as they underwent allogeneic hematopoietic stem cell transplantation with myeloablative conditioning with PBSC as stem cell source were enrolled in the study. Blood samples for the quantitative analysis of circulating EPC levels were drawn at different time points in patients with and without aGvHD. CD34+ VEGFR2/KDR+ CD133+ triple-positive cells identified among CD34+ cells by FACS. EPC were quantified and data are presented as cells/ml whole blood.
Results: Circulating EPC levels were not significantly different in patients with and without aGvHD prior to conditioning (baseline) and at the time of engraftment. However, at diagnosis of aGvHD ≥ grade 2, EPC levels increased whereas in patients without aGvHD the EPC levels remained significantly lower (3021 ± 278 versus 2322 ± 195 cells/ml; $p < 0.001$). Patients with steroid-refractory aGvHD had high levels of EPC throughout. EPC levels fell in responding patients.
Conclusion: Our results demonstrate that the number of circulating EPCs is increased in patients with aGvHD compared to patients without aGvHD.

1. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is used in patients with hematological malignancies [1]. Success of HSCT is limited by the occurrence of acute graft-versus-host disease (GvHD) mediated by host-reactive, donor-derived T cells. The main target organs of the T cells in GvHD are epithelial cells in skin, liver, and intestine and first-line treatment involves high-dose steroids [2]. However steroid refractoriness is observed in ~50% of patients and no established second-line treatments are available. Over the last years, endothelial cells have been increasingly discussed as pathogenetically involved in GvHD [3]. In a study by Penack et al., the anti-VE-cadherin antibody E4G10 inhibited neovascularisation from donor bone marrow (BM)-derived cells without affecting host vascularisation, as well as inhibiting both GvHD and tumor growth and increased survival in a murine allogeneic bone marrow transplantation model, suggesting VE-cadherin as a potential therapeutic target [4]. Furthermore, recent evidence indicates that BM-derived progenitor cells contribute to endothelial cell renewal and repair of GvHD lesions. However, the underlying mechanisms and the contribution of these progenitor cells to the pathophysiology of GvHD are unclear. Endothelial progenitor cells (EPC) presumably bone marrow derived are involved in *vasculogenesis* i.e. the de novo formation of vessels [5,6]. EPCs are a subset of the multipotent CD34+ BM stem cells characterized by an endothelial phenotype. 'Early EPCs' found in the BM, are defined by a typical phenotype, namely by their expression of CD34, CD133 and VEGFR2 (KDR) [7]. In particular, the expression of the cell surface marker CD133 (AC133) on a subpopulation of CD34+ human

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http://dx.doi.org/10.1016/j.leukres.2016.05.011
0145-2126/

Table 1
Patient characteristics.

	(n = 47)
Mean age, years	49 ± 21
Sex (female/male)	17/30
Sex-mismatched allo-HSCT (yes/no)	20/27
Disease (WHO 2008 criteria)	
AML with recurrent genetic abnormalities	17
AML with myelodysplasia-related changes	15
Therapy-related myeloid neoplasms	2
Acute myeloid leukaemia, not otherwise specified	13
Myeloablative conditioning regimen	
Cyclophosphamide/Busulfan (Cy/Bu)	45
Cy/total body irradiation (Cy/TBI)	2
Acute GvHD clinically relevant (≥ grade 2)	22/47

Abbreviations: AML = acute myeloid leukaemia; Bu = Busulfan; Cy = Cyclophosphamide; GvHD = graft-versus-host disease; allo-HSCT = allogeneic hematopoietic stem cell transplantation; TBI = total body irradiation; WHO = World Health Organization.

stem/progenitor BM cells was shown to indicate cells destined for endothelial cell differentiation and angiogenesis. Bone marrow derived circulating EPCs may contribute significantly to neovascularization in a variety of tumors [8]. Cytokines and chemokines released by hypoxic and inflamed tissue have been shown to recruit EPCs and other progenitor cells from the circulation to home to sites of active vessel growth (i.e. neoangiogenesis). Once activated, they migrate and proliferate in order to differentiate to endothelial cells at their place of destination. EPCs are furthermore involved in blood vessel development, in ischemic tissue, following corneal injury or wound healing as well as in tumor growth [9]. The process of differentiation of adult stem/progenitor cells and the *de novo* generation of blood vessels is called *postnatal vasculogenesis* [5]. Further GvHD is associated with transplant-associated thrombotic microangiopathy (TAM) [10]. TAM is characterized by systemic or intrarenal platelet aggregation and a microangiopathic hemolytic anemia, with red blood cell fragmentation and a negative direct antiglobulin test. Increased platelet consumption leads to thrombocytopenia. Other clinical manifestations include fever, renal dysfunction and neurologic abnormalities as a consequence of systemic perfusion deficits.

The elucidation of the detailed cellular and molecular mechanisms involved may lead to a better understanding of immunoregulation and tissue repair in aGvHD. Furthermore, the hypothesis may offer improved insights into new therapeutic strategies to reduce the severity of GvHD. The aim of the current study is to investigate the involvement and occurrence of EPCs in GvHD in patients after allogeneic HSCT as compared to transplanted patients without GvHD. Both angiogenesis, the sprouting of resident tissue endothelial cells (ECs), and vasculogenesis, the recruitment of BM-derived circulating EPCs, are thought to participate in neovascularization. While EPCs have been often implicated in tumor growth, the biologic significance of EPCs during inflammation and in GvHD is unclear.

2. Patients and methods

2.1. Patients

This prospective single center cohort study was performed according to the regulations of the local ethics committee. The patient characteristics are shown in Table 1. Our cohorts met the following inclusion criteria; all patients (1) had AML in complete remission; (2) had undergone HSCT at our institution between 2012 and 2015; (3) with myeloablative conditioning; (4) had documented none or acute GvHD; (5) diagnosed and graded according to standard criteria. Blood samples for the quantitative analysis of circulating EPC levels were drawn before the start of conditioning (baseline), on the day of neutrophils engraftment (neutrophil engraftment was defined as time to neutrophil count >0.5 × 10^9/l), at diagnosis of aGvHD and at days 0, 3, 7 and 14 after initiation of steroid treatment (in patients with aGvHD) and at similar time points after HSCT in patients without aGvHD (range 1–4 days difference from time points in patients with aGvHD). AML was diagnosed according to the World Health Organization (WHO) criteria [11] and remission status defined according to the guidelines of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in AML [12]. Acute GvHD was diagnosed based on clinical symptoms and/or skin, oral mucosa, liver, or gut biopsy and disease severity scored using published consensus criteria [13,14]. GvHD prophylaxis administered along with the myeloablative conditioning regimens was cyclosporine A and methotrexate as well as anti-thymocyte globulin (ATG) in the case of an unrelated donor and in matched related donors ≥40 years old, according to institutional standards. Corticosteroid treatment (methylprednisolone, i.v., 2 mg/kg per day) was started at diagnosis of grade ≥2 aGvHD. Cyclosporine A was continued in patients with aGvHD.

Failure of treatment (corticosteroid resistance) is defined as no response after 7 days of treatment or clear progression after 5 days [15]. TAM was defined as evidence of hemolysis in the presence of schistocytes in the blood smear [10]. Blood smears were analyzed manually twice weekly, and parameters of hemolysis were measured daily. Hemolysis was defined arbitrarily as the combination of LDH ≥ 300 U/l, bilirubin ≥ 25 μmol/l and a decrease in hemoglobin ≥ 10 g/l. Schistocytes ≥ 2–5/hpf were used as the minimum criterion for the diagnosis of TAM. Diagnostic criteria did not include thrombocytopenia, because it was almost universal, and neurological or renal dysfunction, as these were more varied.

2.2. Isolation of mononuclear cells, CD34+ selection and flow cytometry

To quantify the content of circulating EPCs mononuclear cells (MNCs) were isolated from the peripheral blood using Ficoll density gradient centrifugation [16]. For CD34 isolation, the CD34 microbead system from Miltenyi was used. For FACS analysis, 100,000 CD34+ cells was acquired and scored. The following antibodies were used: VEGFR2 (KDR), CD133, CD34 and CD45. CD34+ cells were gated within the defined MNC cell fraction defined by forward-sideward scatter analysis. The population of interest was further defined by dual expression of CD34+/VEGFR2+ (KDR) or CD34+/CD133+. EPC was identified as CD34+VEGFR2/KDR+CD133+ triple-positive cells among CD34+ cells and quantified accordingly by the flow cytometry analysis. Data are presented as EPC cells/ml whole blood.

2.3. Statistical analysis

Statistical analyses, including distribution analysis and descriptive statistics, were performed with IBM SPSS Statistics 22.0. Comparisons were performed using the Kruskal-Wallis and Mann-Whitney *U* (MWU) tests. Differences were considered statistically significant at $P < 0.05$; two-sided tests were used throughout the analysis.

3. Results

3.1. Patients' baseline clinical characteristics and EPC levels

Patients' baseline characteristics, with the distribution of cases according to the 2008 WHO classification, are presented in Table 1. The donors were matched siblings (n = 25) or matched unrelated