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Association between an Impaired Bone Marrow Vascular Microenvironment and Prolonged Isolated Thrombocytopenia after Allogeneic Hematopoietic Stem Cell Transplantation

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A B S T R A C T

Prolonged isolated thrombocytopenia (PT) is a serious complication after allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, it remains unclear whether abnormalities of the bone marrow (BM) microenvironment are involved in the pathogenesis of PT. This prospective, nested case-control study included 20 patients with PT, 40 matched patients with good graft function (GGF) after allo-HSCT, and 16 healthy donors (HDs). Cellular elements of the BM microenvironment, including BM endothelial cells (BMECs), perivascular cells, and endosteal cells, were analyzed via flow cytometry and via hematoxylin-eosin and immunohistochemical staining in situ. Moreover, stromal-derived factor 1 (SDF-1) and vascular endothelial growth factor (VEGF) were measured in the plasma of BM via an enzyme-linked immunosorbent assay. No significant differences in endosteal cells (15 per high-power field [hpf] versus 16 per hpf versus 20 per hpf, $P > .05$) were demonstrated among the patients with PT, GGF, and the HDs. The PT patients exhibited remarkable decreases in cellular elements of the vascular microenvironment, including BMECs (.01% versus .18% versus .20%, $P < .0001$) and perivascular cells (.01% versus .12% versus .13%, $P < .0001$), compared with the GGF allo-HSCT recipients and the HDs, respectively. Moreover, significantly lower levels of SDF-1 (3163 pg/mL versus 3928 pg/mL, $P = .0002$) and VEGF (56 pg/mL versus 123 pg/mL, $P < .0001$) were found in the BM plasma of the PT patients compared with the BM of the GGF patients. A multivariate analysis revealed that BMECs (odds ratio [OR] = 171.57, $P = .002$) and cytomegalovirus infection after HSCT (OR = 4.35, $P = .009$) were independent risk factors for PT. Our data suggested that an impaired BM vascular microenvironment and megakaryocyte-active factors may contribute to the occurrence of PT after HSCT.

INTRODUCTION

Prolonged isolated thrombocytopenia (PT), which is defined as the engraftment of all peripheral blood cell lines other than a platelet (PLT) count $\leq 20 \times 10^9$/L or dependence on PLT transfusions for more than 90 days after allogeneic hematopoietic stem cell transplantation (allo-HSCT) [1,2], remains a serious complication after allo-HSCT. Several risk factors, including the source of the stem cells, the doses of infused $CD34^+$ cells, disease status, graft-versus-host disease (GVHD), and cytomegalovirus (CMV) infection, have been proposed to be associated with PT after allo-HSCT [3-6]. However, the underlying mechanisms remain to be elucidated.

Emerging evidence from mouse studies has suggested that effective hematopoiesis depends on a particular bone marrow (BM) microenvironment in which hematopoietic stem cells (HSCs) reside [7,8]. BM endothelial cells (BMECs), endosteal cells, and perivascular cells have been identified as key supporting elements for HSCs in the murine BM microenvironment [9-11]. We recently established a reliable assay for the anatomic and phenotypic detection of the human equivalents of the above-described murine BM microenvironment elements [12]. Moreover, the frequency of the aforementioned elements has been demonstrated to be remarkably reduced in patients with secondary poor graft function (PGF) compared with patients with good graft function (GGF) and healthy donors (HDs), indicating that the impairment of the BM microenvironment contributes to the occurrence of secondary PGF after HSCT [12].

In mice, the cross-talk between megakaryocytes (MKs) and BMECs in the BM vascular microenvironment regulates MKs maturation and thrombopoiesis [13,14]. Therefore, we hypothesized that an impaired BM microenvironment may hamper MKs maturation, possibly translating to the occurrence of PT after HSCT. To confirm our hypothesis, a prospective, nested case-control study was conducted to evaluate whether the levels of the above-described components of the BM microenvironment [12] and MK-active factors [14] in allo-HSCT patients with PT differed from those in patients with GGF or in HDs. Our aim was to provide new insights into the pathogenesis underlying PT after allo-HSCT.

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PATIENTS AND METHODS

Patients and Controls

A prospective, nested case-control study was designed. Cases were identified from a cohort of 418 patients who underwent allo-HSCT for hematologic malignancies between October 1, 2012 and September 30, 2013 at the Peking University Institute of Hematology. A total of 20 patients who had developed PT after allo-HSCT were eligible for inclusion. For each case, 2 matched controls with GGF (n = 40) were randomly selected from the same cohort of patients undergoing treatment at the time that PT occurred in the cases. Using a risk set sampling design [15], the controls were matched according to the following criteria: age at HSCT (±1 years), pre-HSCT cycles of chemotherapy (±1 cycle), disease status at HSCT, and the duration between HSCT and the evaluation of the BM microenvironment (±5 days). The characteristics of the PT cases and controls are summarized in Table 1.

BM samples from 16 HDs were used as healthy control specimens. The HDs consisted of 8 males and 8 females, and their ages ranged from 18 to 50 years (median, 27 years). The study was approved by the ethics committee of Peking University People's Hospital, and written informed consent was obtained from all the subjects before study entry, in accordance with the Declaration of Helsinki.

Clinical Definitions and Evaluation

Hematopoietic engraftment after transplantation was defined as the reconstitution of both neutrophil and PLT numbers. *Neutrophil reconstitution* was defined as occurring during the first 3 consecutive days with an absolute neutrophil count (ANC) $> .5 \times 10^9$/L, and *PLT reconstitution* was defined as the first time that PLT levels reached $> 20 \times 10^9$/L for 7 consecutive days.

GGF was defined as persistent successful engraftment (ANC $> .5 \times 10^9$/L for 3 consecutive days, PLT counts $> 20 \times 10^9$/L for 7 consecutive days, and hemoglobin [Hb] levels >70 g/L without transfusion support) beyond 28 days after HSCT [12]. *PT* was defined as the engraftment of all peripheral blood cell lines other than a PLT count $\leq 20 \times 10^9$/L or dependence on PLT transfusions for more than 90 days after allo-HSCT [1,2] in the presence of complete donor chimerism. Patients with evidence of PGF [12] or hematologic relapse within 90 days after transplantation were excluded.

Chimerism analyses were performed using DNA fingerprinting for short tandem repeats in blood samples and/or fluorescence in situ chromosomal hybridization in BM samples. *Full donor chimerism* was defined as the failure to detect recipient hematopoietic or lymphoid cells using either of the above methods. The underlying diseases were further categorized as standard-risk or high-risk. *Standard-risk patients* were defined as patients in the first or second complete remission of acute leukemia, patients in the first chronic phase of chronic myelocytic leukemia, or patients with myelodysplastic syndrome. All other patients were classified as high-risk. *Hematologic relapse* was defined as the reappearance of blasts in the blood, BM (>5%) or any extramedullary site after complete remission and was determined using common morphological criteria. GVHD was scored as acute or chronic based on published criteria [16,17].

Transplantation Protocols

Donor selection, HLA typing, stem cell harvesting, conditioning therapy, and GVHD prophylaxis were conducted as previously described [12,18-21]. The grafts that were transplanted were both rh granulocyte colony–stimulating factor–mobilized peripheral blood stem cells and BM stem cells from HLA-matched or HLA-mismatched sibling donors.

All allo-HSCT patients were screened for CMV serostatus before transplantation. Weekly real-time quantitative PCR for the detection of CMV DNA was performed to detect CMV reactivation in the blood after allo-HSCT. CMV infections were treated with ganciclovir or foscarnet, as previously described [22].

Immunophenotypic Analysis of Cellular Components in the BM Microenvironment

As previously reported [12,23,24], the BMECs and perivascular cells in the PT patients, GGF patients, and HDs were analyzed using flow cytometry. In brief, 5 mL of fresh BM was stained with mouse antihuman CD45, CD34, CD146, and vascular endothelial growth factor receptor 2 (VEGFR2) monoclonal antibodies (BD Biosciences, San Jose, CA), and staining was detected using a FACSCalibur (BD Biosciences).

Histological Analysis of the BM Microenvironment

Bone marrow trephine biopsies (BMB) were obtained from 20 PT patients, 40 matched GGF patients, and 16 HDs. H & E staining and immunohistochemistry (IHC) with rabbit antihuman osteopontin, CD34, and CD146 (Abcam, Cambridge, MA) were performed on each BMB section obtained from the patients and controls, as previously described [12]. Each section was examined using light microscopy (Axiovert 200; Carl Zeiss, Jena, Germany).

Methods of Assessment in Histological Analysis

BMB cellularity was assessed via visual examination and categorized into 3 groups [25]. MKs were identified as large cells with blue/gray cytoplasm, which often contained large, multilobed nuclei. MKs were quantified as the number per high-power field (hpf), and endosteal cells were quantified as the number of osteopontin-positive cells on the line of the trabecular bone per hpf. The number of CD146-positive perivascular cells per microvessel was counted. The BM microvessel density in BMB sections was enumerated using IHC with a CD34-reactive monoclonal antibody, according to Perez-Atayde et al. [26]. The median number of vessels per trabecular bone was recorded. The data from the routinely stained H & E sections were compared with the information obtained from IHC. The enumerations were performed by 2 blinded observers.

Enzyme-linked Immunosorbent Assay (ELISA) to Detect Megakaryocyte-active Factors

The levels of stromal-derived factor 1 (SDF-1) and vascular endothelial growth factor (VEGF) in the BM plasma samples from PT and GGF patients were quantified using a Quantikine Human Immunoassay kit (R&D Systems, Minneapolis, MN; and Abcam, Cambridge, MA) according to the manufacturer's protocol. BM plasma samples from HDs were used as a normal control. Briefly, EDTA-anticoagulated BM was centrifuged at 1000 g for 15 minutes within 30 minutes of collection. An additional centrifugation step of the separated plasma at 10,000 g for 10 minutes at 4°C was performed to obtain platelet-poor plasma, which was then aliquoted and stored at −80°C until testing. All the samples were analyzed in duplicate. The absorbance was measured using a Model 680 microplate reader (Bio-Rad, Berkeley, CA). The personnel who performed the ELISA assays were blinded to the clinical background of the samples.

Statistical Analysis

The characteristics of the patients in the PT and GGF groups were compared using the chi-square test for categorical variables and the Mann-Whitney U test for continuous variables. The statistical analyses were performed using a 1-way analysis of variance (ANOVA) to compare the 3 groups. Factors with $P < .10$, as determined via a univariate logistic analysis, were included in the multivariate logistic regression analysis. Factors with $P < .05$ were considered to be independently associated with the occurrence of PT. All statistical procedures were performed using the SPSS 16.0 (IBM, Armonk, NY) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) software packages, and $P < .05$ was considered statistically significant.

RESULTS

Patient Characteristics

Twenty patients with PT and 40 matched patients with GGF after allo-HSCT were enrolled in this study. To minimize the potential influence of the length of time since allo-HSCT, the BM microenvironments of the PT and GGF patients were evaluated at similar median times after allo-HSCT (95 days versus 93 days, $P > .05$). Moreover, PCR DNA fingerprinting of the short tandem repeats of the recipient BM cells was used to confirm 100% donor chimerism in these patients.

As shown in Table 1, there were no significant differences between the allo-HSCT patients with PT and those with GGF ($P > .05$) in demographic and clinical characteristics, including age, gender, underlying disease, disease status before transplantation, median time from diagnosis to transplantation, source of stem cells, transplanted total nucleated cell dose, $CD34^+$ cell dose, donor HLA match, sex/ABO mismatch, pre-HSCT chemotherapy cycles, conditioning, history of GVHD, and duration of anti-CMV therapy. However, a larger proportion of PT patients had a history of CMV infection shortly after allo-HSCT compared with the GGF patients ($P = .02$).