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# Prolonged Thrombocytopenia Following Allogeneic Hematopoietic Stem Cell Transplantation and Its Association with a Reduction in Ploidy and an Immaturation of Megakaryocytes

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Prolonged thrombocytopenia is a frequent complication after allogeneic hematopoietic stem cell transplantation (allo-HSCT); however, its pathogenesis has remained obscure. In the present study, we used flow cytometry to determine the frequency of bone marrow megakaryocytes (MKs) and MK ploidy distributions in allo-HSCT recipients with or without prolonged thrombocytopenia (n = 32 and 27, respectively) and healthy volunteers (n = 13). In addition, the expression of c-Mpl in MKs was measured. The results indicate that the proportions of MKs in marrow mononuclear cells or the percentages of $CD110^+$ MKs in total MKs did not significantly differ between the 3 groups; however, in a comparison of nonthrombocytopenic allo-HSCT recipients to healthy volunteers, the allo-HSCT patients who had prolonged thrombocytopenia exhibited significant shifts toward low ploidy cells (left shift), which were accompanied by a marked increase in ≤8N cells ($P = .036$ and $P < .001$, respectively) and significant decreases in 16N cells ($P < .001$ and $P < .001$, respectively) and ≥32N cells ($P = .01$ and $P < 001$, respectively). These results indicate that there were more immature MKs in allo-HSCT recipients who had prolonged thrombocytopenia, in comparison to nonthrombocytopenic allo-HSCT recipients and healthy volunteers. We conclude that prolonged thrombocytopenia and slow platelet engraftment after allo-HSCT may be related to a reduction in ploidy and an immaturation of MKs.

*Biol Blood Marrow Transplant 17: 274-280 (2011)* © *2011 American Society for Blood and Marrow Transplantation*

**KEY WORDS:** Thrombocytopenia, Megakaryocyte, Ploidy, Allogeneic hematopoietic stem cell transplantation

## INTRODUCTION

Prolonged thrombocytopenia, which is defined as a recovery of all peripheral blood cell lines aside from consistently low platelet counts after transplantation for more than 3 months, is a frequent complication after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Severe thrombocytopenia necessitates platelet transfusion for the management and prevention of bleeding and influences the therapeutic effects and prognosis of transplantation. Kim et al. [1] have identified increased thrombocytopenia-related mortality and infection rates in thrombocytopenia patients after transplantation, wherein low platelet counts on the 60th day after transplantation was an independent risk factor for poor allo-HSCT patient prognosis [2]. Additionally, other studies have described poor prognoses in patients with thrombocytopenia 90 days after allogeneic bone marrow transplantation [3,4].

The major causes of thrombocytopenia include accelerated peripheral platelet destruction by antiplatelet antibodies and the insufficient production of platelets from marrow megakaryocytes (MKs). In previous studies, both increased platelet turnover and impaired thrombopoiesis have been reported to be involved in the development of prolonged thrombocytopenia after HSCT, wherein the latter mechanism plays a predominant role [5,6]; however, until recently, little has been understood regarding the development and maturation of the immediate precursors of platelets, which are the marrow MKs, in post-allo-HSCT patients. The primary feature of MK maturation is the development of a single,

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*Financial disclosure:* See Acknowledgments on page 279.

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Received July 7, 2010; accepted September 12, 2010

1083-8791/$36.00
doi:10.1016/j.bbmt.2010.09.007

large, lobulated, and polyploid nucleus. Mature MKs cease to proliferate but continue to increase their DNA content without undergoing the late stages of mitosis [7-9]. To our knowledge, no study has evaluated the ploidy pattern in MKs in post-allo-HSCT patients.

C-Mpl (CD110), which is expressed on the surfaces of MKs and MK precursors, is a receptor for thrombopoietin (TPO). TPO has been identified to be a key cytokine for megakaryogenesis and thrombopoiesis because it binds to c-Mpl (CD110). Yamazaki et al. [6] have found that plasma TPO was significantly increased in HSCT recipients with thrombocytopenia in comparison to those without; however, no study has focused on c-Mpl (CD110) expression in MKs in post-allo-HSCT patients.

The aim of the present study was to evaluate whether the ploidy distribution pattern in MKs and the level of c-Mpl expression on the surface of MKs in allo-HSCT patients with prolonged thrombocytopenia differed from those in allo-HSCT patients without prolonged thrombocytopenia or healthy volunteers.

## PATIENTS AND METHODS

### Patients and Controls

Prolonged thrombocytopenia was defined as a platelet count $\leq 80 \times 10^9$/L for more than 3 months after HSCT, recovery of all other cell counts, and no apparent cause for thrombocytopenia, such as engraftment failure, recurrence of the underlying malignancy, microangiopathy, or drugs. We studied 32 allo-HSCT recipients who had prolonged thrombocytopenia. As a control, we selected 27 allo-HSCT recipients who did not have prolonged thrombocytopenia after day 90 and who had recovered all other cell counts. To minimize the potential influence of the length of time after allo-HSCT, all of the enrolled patients had their bone marrow tested approximately 3 months after allo-HSCT (median +88 days, range: +83 to +120 days). Patients were excluded if they died, had disease recurrence within 90 days of transplantation, or if they initially failed neutrophil engraftment $>0.5 \times 10^9$/L. Clinical characteristics were matched between the study and control groups when possible. All of these patients underwent allo-HSCT for various hematologic malignancies or nonmalignancies between October of 2008 and May of 2009 at Peking University People's Hospital.

Bone marrow samples from 13 healthy volunteers were used as healthy control subjects. The 13 healthy controls consisted of 6 males and 7 females, and their ages ranged from 16 to 51 years (median: 28 years).

Bone marrow samples were obtained after the patients and control subjects gave their written informed consent. The institutional review board of the Peking University Institute of Hematology and the ethics committee of Peking University People's Hospital approved this study.

### allo-HSCT Procedure

The conditioning therapy for HLA-mismatched and unrelated matched HSCT patients included modified BUCY2 plus ATG (thymoglobulin), which consisted of intravenous cytarabine (4 g/m$^2$/day) on days –10 to –9, intravenous busulfan (3.2 mg/kg/day) on days –8 to –6, intravenous cyclophosphamide (1.8 g/m$^2$/day) on days –5 to –4, oral Me-CCNU (250 mg/m$^2$) once on day –3; and intravenous antithymocyte globulin (ATG) (2.5 mg/kg/day; Sang Stat, Lyon, France) on days –5 to –2. In matched sibling transplantations, patients received a regimen that was identical to that of HLA-mismatched patients without ATG except they orally received hydroxycarbamide (80 mg/kg) on day –10 and a lower dose of cytarabine (2 g/m$^2$/day) on day –9. The transplanted grafts were both granulocyte colony-stimulating factor-mobilized peripheral blood stem cells (PBSCs) and bone marrow (BM) cells from matched sibling or HLA-mismatched sibling donors or PBSCs from unrelated donors. Post allo-HSCT, filgrastim rh-granulocyte-colony stimulating factor (rhG-CSF) was subcutaneously administered to HLA-mismatched and unrelated matched HSCT patients at 5 μg/kg/day from day +6 after transplantation until the neutrophil count reached $0.5 \times 10^9$ cells/L for 3 consecutive days; however, in matched sibling transplantations, filgrastim (rhG-CSF) was not used post-allo-HSCT.

All patients received cyclosporine A (CsA), mycophenolate mofetil (MMF), and short-term methotrexate for graft-versus-host disease (GVHD) prophylaxis [10,11]. Methotrexate (MTX) was intravenously administered at 15 mg/m$^2$ on day +1, at 10 mg/m$^2$ on days +3 and +6 after all of the transplantations, and again at 10 mg/m$^2$ on day +11 after HLA-mismatched and unrelated matched HSCT. MMF was discontinued upon engraftment after matched sibling HSCT, whereas in the patients with HLA-mismatched and unrelated matched HSCT, MMF was tapered from 1 g/day to 0.5 g/day on day 30 and was discontinued over days 45 to 60 based on the presence or absence of severe GVHD, infectious diseases, or risk of relapse. Acute and chronic GVHD (aGVHD, cGVHD) were defined according to published criteria [12,13].

All allo-HSCT patients were screened pretransplantation for cytomegalovirus (CMV) serostatus. Weekly polymerase chain reaction (PCR) (Roche, Amplicor, Indianapolis, IN, USA) was employed to survey CMV reactivation in the blood from the time of transplantation to 100 days afterward.