Thrombosis Research 139 (2016) 1–9

Contents lists available at ScienceDirect

Thrombosis Research

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

Full Length Article

Increased prostacyclin levels inhibit the aggregation and activation of platelets *via* the PI3K–AKT pathway in prolonged isolated thrombocytopenia after allogeneic hematopoietic stem cell transplantation

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ARTICLE INFO

Article history:
Received 27 September 2015
Received in revised form 28 November 2015
Accepted 2 January 2016
Available online 5 January 2016

Keywords:
Prostacyclin
Hematopoietic stem cell transplantation
Prolonged isolated thrombocytopenia
Platelet activation
Platelet aggregation

ABSTRACT

Objectives: The aim of this study was to investigate the role of prostacyclin (PGI_2) in prolonged isolated thrombocytopenia (PT) following allogeneic hematopoietic stem cell transplantation (allo-HSCT) and the effect of PGI_2 on the activation and aggregation of platelets in PT.
Methods: We enrolled 37 patients with PT and 36 controls following allo-HSCT in this study. Platelet aggregation and activation and PGI_2 levels were measured. Endothelial progenitor cells (EPCs) from either PT or control patients were cultured *ex vivo* with serum from either PT or control patients. PGI_2 secretions were then measured. PGI_2 was added to the platelets *ex vivo*, and platelet aggregation and activation and PI3K/Akt phosphorylation were analyzed.
Results: A higher PGI_2 level was observed in the PT patients. The activation and aggregation of platelets were significantly lower in the PT patients. EPCs from PT patients cultured in PT serum secreted higher levels of PGI_2, and PGI_2 inhibited platelet activation and aggregation in a concentration-dependent manner *ex vivo*. PI3K/Akt phosphorylation of platelets was regulated by PGI_2 after allo-HSCT. Disease status, serum PGI_2 level and platelet aggregation were independent risk factors in patients with PT after allo-HSCT.
Conclusions: Higher PGI_2 levels and lower platelet activation and aggregation occurred simultaneously in PT patients. PGI_2 inhibited platelet activation and aggregation, probably by regulating the phosphorylation of PI3K/Akt.

1. Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective treatment for malignant hematological diseases, whereas prolonged isolated thrombocytopenia following allo-HSCT can dramatically decrease patient survival [1]. It has been reported the incidence is approximately 5–37% [1,2]. Prolonged isolated thrombocytopenia (PT) is defined as a recovery of all peripheral blood cell lines aside from consistently low platelet counts after transplantation for more than 3 months [3]. Kim et al. [4] has already proved that, in patients with PT after allo-HSCT, the mortality rate related with thrombocytopenia and the infection incidence are higher. PT contributes to an increased risk of bleeding, and bleeding was significantly associated with reduced survival [5]. However, bleeding has also been associated with acquired hepatic dysfunction and specific end-organ toxicities, such as hemorrhagic cystitis, diffuse alveolar hemorrhage, and graft *versus* host disease (GVHD)-associated gastrointestinal bleeding [6,7]. Among patients undergoing HSCT, 15–24% experience clinically significant bleeding complications [6,7]. It is well accepted that the main causes of hemorrhage are thrombocytopenia, abnormal coagulation and injury of endothelial cells [8–10].

However, the platelet activation and aggregation in PT following allo-HSCT remain unknown. It has been investigated that patients following allo-HSCT had wide damage to endothelial cells [8,11]. In *ex vivo* experiments, endothelial progenitor cells (EPCs) could inhibit platelet activation and aggregation *via* the secretion of prostacyclin (PGI_2), a well-known antiplatelet agent, by increasing the level

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http://dx.doi.org/10.1016/j.thromres.2016.01.003
0049-3848/

of cAMP [12,13]. In light of the antiplatelet capability of PGI_2, we conducted the following study to explore the function of platelets in PT.

We hypothesized that an increase in PGI_2 might contribute to the inhibition of platelet activation and aggregation. We found that PT was associated with higher levels of PGI_2 and lower platelet aggregation and activation *via* the PI3K/AKT pathway. These results implied, for the first time, that the regulation of platelet aggregation and activation may be one of the characteristics of PT.

2. Patients and methods

2.1. Clinical definitions

PT was defined as a platelet count ≤100 × 10^9/L for more than 3 months after HSCT, with recovery of all the other cell counts and no apparent cause for thrombocytopenia including engraftment failure, recurrence of the underlying malignancy, donor lymphocyte infusion (DLI) or death [2,3,14,15]. Neutrophil engraftment after transplantation was defined as an absolute neutrophil count (ANC) in excess of 0.5 × 10^9/L for 3 consecutive days. The first of these 3 consecutive days was considered to be the day of engraftment. The day of platelet engraftment was defined as the first day of 7 consecutive days in which the patient had a platelet count of >20 × 10^9/L and did not receive a platelet transfusion [16,17]. Patients were categorized as "standard risk" if they were in their first or second complete remission (CR1 or CR2) of acute leukemia (AL) without [t (9; 22) (q34; q11)], if they were in the chronic phase of chronic myeloid leukemia (CML), or if they had other types of hematological malignancies. Patients were classified as "high risk" if they had AL with [t (9; 22) (q34; q11)] regardless of the disease stage, if they had AL in its third complete remission (CR3) or greater, if they were in non-remission regardless of cytogenetics, or if they had CML beyond the first chronic phase [16,18]. Hematologic relapse was defined as the reappearance of blasts in the blood or BM (>5%) or any extra medullary site after complete remission in tests performed using common morphological criteria. GVHD was classified based on published criteria [19].

2.2. Patients and controls

The study protocol was approved by the Ethics Committee of Peking University People's Hospital. Cases were identified from a cohort of 133 patients who underwent allo-HSCT between Nov 1, 2013, and Feb 28, 2014, at Peking University People's Hospital Institute of Hematology. From these patients, 73 consecutive patients were enrolled in this study. Thirty-seven patients were identified developed PT. They were further divided into two groups according to platelet count of 50 × 10^9/L at the 3rd month after allo-HSCT, in which there were 14 patients with platelet counts less than 50 × 10^9/L and 23 with platelet counts equal to or greater than 50 × 10^9/L. Thirty-six recipients without PT were enrolled as controls. They were routinely tested before sampling and confirmed complete donor chimerism status. All of the patients had samples obtained approximately 3 months after allo-HSCT (median: +94 d, range: +82 d to +132 d). Patients were excluded if they died, had disease recurrence or did not acquire cell engraftment between the day after transplantation and the sampling day [16]. Clinical characteristics were matched between the study and control groups, as shown in Table 1. All of the patients provided their written informed consent.

Table 1
Clinical characteristic of the patients.

Characteristics	Prolonged isolated thrombocytopenia		*P* value
	Present (n = 37)	Absent (n = 36)	
Age, median (range)	34 (23–44)	31 (23–43)	0.604
Male (%)	19 (52.8%)	17 (47.2%)	0.724
Underlying disease			
AML	18 (48.6%)	21 (58.3%)	0.311
ALL	7 (18.9%)	10 (27.8%)	
MDS	6 (16.2%)	1 (2.8%)	
CML	3 (8.1%)	1 (2.8%)	
SAA	2 (5.4%)	0 (0.0%)	
NHL	1 (2.7%)	3 (8.3%)	
Donor type			
Haplo-identical	29 (78.4%)	22 (61.1%)	0.186
HLA-matched			
Sibling	7 (18.9%)	14 (38.9%)	
Unrelated	1 (2.7%)	0 (0%)	
ABO matched (%)	14 (37.8%)	12 (33.3%)	0.688
Transplanted total nucleated cell dose, ×10^8/kg, median (range)	7.1 (6.0–7.9)	7.2 (6.8–8.5)	0.257
Transplanted CD34+ cell dose, ×10^6/kg, median (range)	2.0 (1.3–2.8)	2.4 (1.8–4.1)	0.708
Engraftment time			
WBC	14 (11–16)	13 (11–15)	0.385
PLT	22 (13–34)	13 (11–17)	0.000
Complications			
Acute GVHD	16 (43.2%)	20 (55.5%)	1.000
Extensive chronic GVHD	2 (5.4%)	1 (2.8%)	0.572
History of CMV infection	24 (64.9%)	12 (33.3%)	0.067

ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome; CML, chronic myelogenous leukemia; SAA, severe aplastic anemia; WBC, white blood cell; PLT, platelet; GVHD, graft *versus* host disease; CMV, cytomegalovirus.

2.3. Transplantation procedure and treatment

HLA-mismatched and unrelated matched HSCT patients received a modified busulfan/cyclophosphamide (BU/CY) plus thymoglobulin (ATG) regimen, which consisted of intravenous cytarabine (4 g/m^2·d), on d(−10) to d(−9), intravenous busulfan (3.2 mg/kg/d), on d(−8) to d(−6), intravenous cyclophosphamide (1.8 g/m^2·d), on d(−5) to d(−4), oral Me-CCNU (250 mg/m^2), on d(−3), and intravenous antithymocyte globulin (ATG) (2.5 mg/kg/d; Sang Stat, Lyon, France) on d(−5) to d(−2). Patients who received matched sibling transplantations underwent a regimen that was identical to that of the HLA-mismatched patients without ATG except they received oral hydroxycarbamide (80 mg/kg), on d (−10), and a lower dose of cytarabine (2 g/m^2/d), on d (−9). All of the donors underwent granulocyte colony-stimulating factor (G-CSF)-mobilizing treatment. The recipients with sibling donors received bone marrow (BM) and peripheral blood stem cells (PBSCs). Those with unrelated donors received only PBSCs. Cyclosporine A, mycophenolatemofetil, and short-term methotrexate were used for post-HSCT GVHD prophylaxis. After allo-HSCT, recombinant human G-CSF (rhG-CSF) was administered to HLA-mismatched and unrelated matched allo-HSCT recipients at 5 μg/kg/d from d (+6) until the neutrophil count reached 0.5 × 10^9/L for 3 consecutive days. rhG-CSF was not used after allo-HSCT with matched sibling transplants [3,20,21].

2.4. Platelet aggregation

Platelet aggregation was evaluated as previously described [22]. Aggregation was monitored by measuring light transmission with an aggregometer (Chrono-log, Havertown, PA, USA). The washed platelets were prepared by centrifugation (100 g, 10 min, 20 °C), adjusted to the proper number (10^8/mL) and preincubated at 37 °C for 2 min with either iloprost (a stable analog of prostaglandin, PGI_2) or vehicle. Then, they were stimulated with 10 μM adenosine diphosphate (ADP) plus 50 μM epinephrine. The mixture was further incubated for 5 min with stirring of 1200 rpm.