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A mouse model to study thrombotic complications of thalassemia

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ABSTRACT

Patients with β-thalassemia major and mainly intermedia have an increased risk for developing venous and arterial thrombosis which may be related to circulating pathological red blood cells (RBC) and continuous platelet activation. In the present study we used a modified thalassemic mice model in conjunction with a "real-time" carotid thrombus formation procedure to investigate thrombotic complications of thalassemia. Heterozygous Th3/+ mice, which lack one copy of their β-major and β-minor globin genes, exhibit anomalies in RBC size and shape, chronic anemia and splenomegaly which recapitulate the phenotype of human β-thalassemia intermedia. Flow cytometry measurements showed higher reactive oxygen species generation, indicating oxidative stress, in platelets and RBC of the thalassemic mice compared with wild type mice concomitant with an increase in reduced glutathione content which may represent a compensatory response to oxidative stress, and exposed phosphatidylserine which indicates platelet activation. To elucidate the effect of thalassemia on the development of arterial thrombosis, we studied photochemical-induced real-time thrombus formation in the carotid artery of these mice. The results indicated a significantly shorter "time to occlusion" in the thalassemic mice compared to wild type mice, which was prolonged following in vivo aspirin treatment. We suggest that this mouse model may contribute to our understanding of platelet activation and the hypercoagulable state in thalassemia and lay foundations to screening of anti-platelet drugs as well as anti-oxidants as possible therapeutics for prevention of thrombosis in thalassemia patients.

Introduction

Beta (β)-thalassemia major is a severe hereditary anemia caused by a partial or complete absence of the β-globin chains of the hemoglobin molecule, resulting in absence or deficiency in hemoglobin A. Increased life expectancy due to improved therapy is associated with the development of complications in adult patients which were not recognized earlier, such as hypercoagulable states, including deep venous thrombosis and pulmonary embolism as well as arterial thrombosis and stroke [1]. An increased circulating number of pathological RBC, mainly in splenectomized patients [2] and activated platelets were suggested as potential causes underlying the higher rate of thrombosis in thalassemia. Thus, platelets were shown to express high levels of activation markers [3] and to have a shorter life span due to enhanced consumption [4]. Increased urinary metabolites of thromboxane A_2 and prostaglandin and increased circulating platelet aggregates [5] also suggest a state of chronic platelet activation in these patients.

Recently, we reported an increased platelet adhesion in β-thalassemia, which is correlated with clinical thrombotic events, and might be due to direct contact with the pathological RBC that express abnormally high levels of negatively charged phospholipids, mainly phosphatidylserine (PS), on their membrane [6]. Other factors contributing to the chronic hypercoagulable state in thalassemia include deficiency of coagulation factor inhibitors such as protein C and protein S, cardiac and liver dysfunction and hormonal deficiencies due to iron accumulation in vital organs [7].

Oxidative stress may also contribute to a chronic hypercoagulable state by stimulating platelet activation and aggregation [8] and by inducing PS externalization [9]. We have previously reported oxidative stress in RBC and platelets in thalassemic [10] as well as in other conditions associated with thrombosis such as Paroxysmal Nocturnal Hemoglobinuria [11].

In the present study, we used a thalassemic mice model, Th3/+, which was generated by deletion of the major and minor β-globin genes, so that they have only one copy of each of the adult mouse β -globin genes [12,13]. Mice homozygous for this deletion die in

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uteru, but heterozygotes are viable, but with anemia, abnormal RBC morphology, splenomegaly, and hepatic iron depositions, symptoms similar to those found in patients with β-thalassemia intermedia.

In this study, oxidative stress markers were measured on platelets and RBC of Th3/+ mice and compared to wild-type (wt). In addition, Th3/+ mice were subjected to thrombosis of the carotid artery, using photochemical injury, and the "time to vessel occlusion" was determined. Finally, we used this model to evaluate the effect of treatment with the anti-platelet drug aspirin. The results suggest that anti-platelet drugs as well as anti-oxidants might be used for prevention of thrombosis in thalassemia patients.

Methods

Mice

The transgenic heterozygous thalassemic mice (C57B1/6 Th3/+), kindly donated by S. Rivella (Weill - Cornell Medical College, NY), have targeted mutations in both murine β-globin genes (major and minor) on chromosome one [12,13]. Mice were housed under SPF conditions and were screened for the thalassemia phenotype at 6 weeks of age using peripheral blood smears. Originally, male mice of this transgenic strain were cross-bred with healthy C57B6 females; however, since the liters were very small and fragile, we crossed these males with healthy CB6F1/OlaHsd females. Much larger, sturdy, litters (hybrid vigor) were obtained. We tested the litters for several generations to ensure that the thalassemic phenotype was not affected. Sex- and age-matched wild type (wt) control mice were used in all experiments.

Blood samples were obtained by retro-orbital puncture under anesthesia. Complete blood counts (CBC) were measured on a Coulter counter (LH750 Beckman Coulter Analyzer, Nyon, Switzerland). Blood smears were made using peripheral blood from the tail vein and stained with a Hema-tek 2000 instrument (Bayer healthcare). The protocols for this study were approved by the Institutional Animal Care and Use Committee.

Measurements of Oxidative Stress Markers

Oxidative stress markers were measured in whole blood by flow cytometry as previously described [10,14]. Briefly, reactive oxygen species (ROS) were measured by staining with 2'-7'-dichlorofluorescin diacetate, reduced glutathione (GSH) - by staining with mercury orange and external phosphatidylserine (PS) - by staining with FITC-Annexin V. Following treatments, the cells were analyzed with a Fluorescence-Activated Cell Sorter. RBC and platelets were gated based on their size (forward light scatter) and granularity (side light scatter). The identity of each cell population was verified by staining with antibodies to glycophorin-A and CD41 for RBC and platelets, respectively. The Mean Fluorescence Intensities (MFIs) and the percentages of gated positive cells were calculated using the FACS-equipped CellQuestR software.

Occlusive Thrombus Formation

Photochemical-induced injury to the carotid artery was performed as previously described [15]. The left common carotid artery was isolated, and a vascular flow probe (Transonic Systems) was applied to monitor blood flow. The photosensitizer Rose Bengal (Fisher Scientific Co.) at 10 mg/mL in phosphate-buffered saline, was injected into the vein tail to administer a dose of 50 mg/kg. The mid portion of the carotid artery was then continuously illuminated with a 1.5 mW green laser light (540 nm; Melles Griot, Inc.), and the time required to form an occlusive thrombus, defined as absence of blood flow for 3 minutes or more, was recorded.

Results

Hematological Findings

No differences were noted in weight and survival between the thalassemic (C57B1/6 Th3/$^{+}$X CB6F1/OlaHsd F1 hybrid) mice and their wt counterparts.

Th3/+ mice demonstrated enlarged spleens with average weight of 420 mg compared to 130 mg of wt spleens. Blood smears of Th3/+ mice showed aberrant RBC morphology, including, microcytosis, hypochromia, anisocytosis, poikilocytosis, target cells and reticulocytosis, all characteristics of thalassemia intermedia (Fig. 1a). Their CBC revealed anemia and high platelet count compared to wt controls ($p < 0.0001$ and $p = 0.0072$, respectively). No significant difference was found in white blood cell (WBC) counts between the groups (Fig. 1b).

Oxidative Stress Markers

Flow cytometry measurements showed a significantly higher ROS, GSH and external PS in platelets and RBC from the thalassemic mice as compared to cells from wt mice (Figs. 2, 3). The calculated average of the ROS-MFI values were 95 vs. 65 in platelets ($p = 0.2$), and 30.5 vs.16.6 ($p = 0.018$) in RBC from the thalassemic and wt mice, respectively. Higher content of GSH was found in platelets (48 vs. 27 ($p = 0.0069$)) and RBC (76 vs. 43 ($p = 0.0077$)) of Th3/+ mice compared to wt mice. The platelet activation marker, PS, was higher in platelets of the thalassemic mice compared to wt mice (27% vs. 13%, $p = 0.0089$) and also in RBC (1.5% vs. 0.6%, $p = 0.0087$) (Fig. 3).

Thrombus Formation

To assess the in vivo prothrombotic state, we used an experimental model of real-time thrombus formation in the carotid artery. Following a photochemical injury to the carotid, the time to vascular thrombotic occlusion was measured. The results (Fig. 4) showed shorter time to occlusion in the thalassemic mice (49.1 ± 9 minutes) compared to wt mice (64.6 ± 8 minutes) ($n = 10$ per group; $p < 0.05$). Aspirin treatment, at a single dose of 30 mg/kg by IP injection, prolonged the time to occlusion in wt mice to 72.5 ± 13.06 minutes and to 58.7 ± 8 minutes in th3/+ mice (Fig. 4).

Discussion

Thalassemic patients have an increased risk for developing venous and arterial thrombosis which is due to abnormalities in their RBC and platelet activation [2–7]. The present study proposes a mice model as a tool to study the prothrombotic state in thalassemia. In this model, the mice lack their β^{major} and β^{minor} globin genes (Th3). Mice heterozygous for this deletion (Th3/+), generated by crossing with healthy mice, are viable but exhibit anomalies in RBC size and shape, chronic anemia and splenomegaly (Fig. 1) which recapitulate the phenotype of human β-thalassemia intermedia. Higher platelet counts, compared to WT mice, were also found in these mice, consistent with increased rate of red blood cell turnover and hemolysis. Higher platelet counts could be related to the anemia-mediated increased levels of erythropoietin [16].

Many studies have shown that patients with thalassemia are under chronic oxidative stress [17,18]. The underlying mechanisms are mainly due to hemoglobin instability, a result of the mutation in the globin gene, and iron overload, a consequence of repeated blood transfusions and increased iron uptake from the gastrointestinal tract due to inappropriately low hepcidin expression [19]. Consequently, free intracellular labile iron (LIP) participates in biochemical (Fenton/Haber-Wise) reactions that generate ROS, which when present in excess are cytotoxic. Although the primary etiology of thalassemia is mutations in the globin genes, the consequences of many pathological aspects of the disease are mediated by oxidative stress. Thus, in the erythroid lineage, oxidative