Thrombosis Research 134 (2014) 889–894

Contents lists available at ScienceDirect

Thrombosis Research

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

Regular Article

Platelet activation biomarkers in Berkeley sickle cell mice and the response to prasugrel

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ARTICLE INFO

Article history:
Received 23 May 2014
Received in revised form 14 July 2014
Accepted 22 July 2014
Available online 6 August 2014

Keywords:
Sickle cell disease
Adenine nucleotide
Platelet
$P2Y_{12}$ antagonist
Prasugrel

ABSTRACT

Vaso-occlusive crisis (VOC) is a common complication that occurs in sickle cell disease (SCD) patients. Although underlying mechanisms of VOC remain unclear, platelet activation has been associated with VOC. In the present study, plasma adenine nucleotide measurements using LC-ESI-MS/MS showed that plasma ADP in the Berkeley murine model of SCD was significantly higher (applox. 2.7-fold increase) compared with control mice. Assessment of platelet activation markers using flow cytometry indicated that in SCD mice at steady state (8 weeks old), circulating platelets were partially activated and this tended to increase with age (15 weeks old). The administration of prasugrel, a thienopiridyl $P2Y_{12}$ antagonist, did not affect the activation state of circulating platelets suggesting $P2Y_{12}$ independent mechanism of activation. In this murine SCD model, ex vivo addition of ADP or PAR4 TRAP resulted in further platelet activation as assessed by expression of activated GPIIb/IIIa and P-selectin both at 8 and 15 weeks. In 15 weeks old SCD mice, agonist-induced increases in activation markers were enhanced compared to control mice. Oral administration of prasugrel effectively inhibited ex vivo platelet activation consistent with clinical data in patients with SCD. In conclusion, in the Berkeley murine model of SCD, we found evidence of basal and agonist-stimulated platelet activation which could in part be attenuated by prasugrel. These data are consistent with observations made in patients with SCD and suggest possible utility of this murine model and prasugrel therapy in exploring treatment options for patients with SCD.

Introduction

Sickle cell disease (SCD) results from a single mutation in the β-globin gene. The polymerization of deoxygenated hemoglobin S, containing the mutated β-globin, causes erythrocyte sickling. Patients with SCD suffer periodic acute painful events known as vaso-occlusive crisis (VOC) and eventual end-organ damage from the resulting repeat ischemia-reperfusion insults [1].

Prasugrel, which is a third generation thienopiridyl platelet $P2Y_{12}$ ADP receptor antagonist, has been shown to effectively inhibit platelet activation and aggregation via selective inhibition of platelet $P2Y_{12}$ [2]. Phase 1 and Phase 2 trials of prasugrel have been conducted in adult patients with SCD [3–5], and a Phase 3 trial designed to assess the efficacy of prasugrel in reducing VOC in children with SCD is currently ongoing (ClinicalTrials.gov Identifier: NCT01794000). Notably, prasugrel active metabolite, R-138727, inhibited in vitro ADP-stimulated platelet activation in patients with SCD [6], and prasugrel administration resulted in a significant decrease in the expression of platelet activation biomarkers in patients with SCD [3,4]. Furthermore, in the phase 2 study, prasugrel treatment was associated with a reduction in pain frequency and intensity [5]. In an earlier placebo-controlled double blind study, ticlopidine significantly reduced painful crises in patients with SCD [7]. These results suggest that ADP-induced platelet activation may be a factor contributing to the occurrence of VOC in patients with SCD.

Berkeley sickle cell mice (SCD mice) [8], a murine model of SCD, are genetically modified mice whose murine α-globin and β-globin have been deleted, and in place express human α-, γ-, and δ-globins as well as sickle β-globin. These mice exhibit several characteristics of human SCD including the presence of sickle-shaped erythrocytes [8], hemolytic anemia [9] and ischemic organ failure [10]. Based on the above features, this strain might be considered a useful model for the delineation of the pathogenesis of SCD and to aid in the development of therapeutic modalities for SCD. However, there are only limited reports of platelet studies in this model, at present limited to a description of platelet morphology and responsiveness to protease activated receptor-4 thrombin

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http://dx.doi.org/10.1016/j.thromres.2014.07.035
0049-3848/

receptor activating peptide (PAR4 TRAP) [11]. To our knowledge, there are no reports that describe the effects of antiplatelet drugs in this murine SCD model.

The aim of the present study was to characterize platelet reactivity and its modulation by prasugrel in Berkeley SCD mice. Specifically our determinations included an assessment of the plasma adenine nucleotide concentrations, particularly ADP, elevations of which might underlie the chronic platelet activation in SCD. Using flow cytometry, we examined platelet activation and its modulation by oral administration of prasugrel in SCD mice to investigate the possible role of ADP/$P2Y_{12}$ signaling in platelet activation in SCD.

Materials and Methods

Materials

PAR4 TRAP (AYPGKF-amide) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). ADP was purchased from Sigma-Aldrich Co. and Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). AMP and ATP were purchased from Tokyo Chemical Industry Co. Ltd. Fluorescein isothiocyanate (FITC)-conjugated antibodies for GPIX (rat anti-mouse, Xia.B4) and phycoerythrin (PE)-conjugated antibodies for activated GPIIb/IIIa (rat anti-mouse, JON/A) and P-selectin (rat anti-mouse, Wug.E9) were purchased from Emfret Analytics (Eibelstadt, Germany). Prasugrel hydrochloride (prasugrel) was provided by Ube Industries Ltd. (Ube, Yamaguchi, Japan). Gum arabic was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Animals

The Berkeley sickle cell mice (Tg[Hu-miniLCR$\alpha^{G}\gamma^{A}\gamma\delta\beta^{S}$] $Hba^{-/-}$ $Hbb^{-/-}$) [8] were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred at Charles River Laboratories Japan, Inc. As the control mice, we used the hemizygous sickle cell littermate (Tg[HuminiLCRαGγA$\gamma\delta\beta$S] Hba-/- Hbb+/-) generated by intercrossing α-/-, β+/-, Tg males and α-/-, β+/-, Tg females. All animal studies were approved by the Institutional Animal Care and Use Committee (Ina Research Inc.; Shin Nippon Biomedical Laboratories, Ltd.; NISSEI BILIS Co., Ltd.; and Daiichi Sankyo Co., Ltd.).

Adenine Nucleotides Quantification

Seven-week-old SCD and control mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.). Blood was collected from an abdominal vein into tubes containing ethylenediaminetetraacetic acid (EDTA) and centrifuged at 3000 ×*g* for 10 min at 4 °C to obtain plasma which was immediately aspirated and stored at −80 °C for subsequent analysis. Twenty μL plasma samples or PBS solutions (for standards) were thoroughly mixed with 60 μL of protein precipitate solution (50% acetonitrile/methanol), and then mixed with 20 μL of 30% acetonitrile solution or standard solutions (for standards) and centrifuged at 21900 ×*g* for 15 min at 4 °C. Eighty μL of the supernatant was added to 20 μL of PBS and then mixed for 10 min at room temperature.

Adenine nucleotide concentrations were measured using a LC-ESI-MS/MS system which consisted of an Alliance 2795 Separations Module and Quattro Premier MS/MS system (Waters Corp.). The MassLynx software (version 4.1, Waters Corp.) was applied for data acquisition and processing. Chromatographic resolution was performed on a Hypercarb column (3 μm, 2.1 mm × 150 mm; Thermo Scientific) and the column was maintained at 30 °C. The flow rate was set to 0.3 mL/min. Mobile phase A consisted of H_2O/10 M CH_3COONH_4/10% NH_4OH (1000/0.2/20, v/v/v) and mobile phase B consisted of CH_3CN/10 M CH_3COONH_4/10% NH_4OH (1000/0.2/20, v/v/v). The gradient elution program was as follows: 10% B from 0 to 8 min, 10–90% B from 8 to 13 min, 90% B from 13 to 15 min, and 10% B from 15 to 20 min. For each run, 5 μL of the processed sample was injected. In the MS/MS analysis, ESI was performed in the positive ion mode with a spray voltage of 2500 V. The source temperature was set at 120 °C and the desolvation temperature was set at 450 °C. Collision energy, precursor and product ions were 50 V and 348.1–136.1 *m/z* for AMP, 25 V and 428.1–136 *m/z* for ADP, and 40 V and 508.1–136 *m/z* for ATP, respectively.

Drug Administration

Prasugrel was suspended in 5% (w/v) gum arabic solution. The vehicle (5% gum arabic solution) and the suspension of prasugrel were orally administered to mice in a volume of 10 mL/kg. Doses used for the platelet function studies were based on pilot studies and a previous report [12], both showing effective inhibition of ADP-induced platelet aggregation at the doses used in mice.

Platelet Aggregation

Platelet aggregation in individual platelet-rich plasma (PRP) was measured as previously described [12], with minor modifications. When the platelet counts in PRP were $>20 \times 10^4$/μL, the PRP samples were diluted to 20×10^4/μL with platelet-poor plasma. Platelet aggregation induced by ADP (final concentration of 20 μM) was measured for 10 min with a platelet aggregometer (PRP313M, IMI Co. Ltd.) and maximum platelet aggregation (%) was determined.

Flow Cytometric and Hematological Analyses

Animals were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) at 4 h after the final administration of the test article. Blood was collected from an abdominal vein into tubes containing 1/9 (v/v) volume of 3.8% sodium citrate solution, and used for flow cytometric and hematological analyses. Hematological parameters were measured on citrated whole blood samples by an ADVIA120 (Bayer Diagnostics). Flow cytometric analyses were conducted according to the method of Frelinger et al. [6] with the following modifications: For the analysis of activated GPIIb/IIIa, citrated whole blood samples (10 μL) were added to a mixture (20 μL) of FITC-conjugated anti-GPIX for platelet identification, PE-conjugated anti-activated GPIIb/IIIa, and saline or ADP (final concentration of 20 μM). For the analysis of P-selectin, a citrated whole blood sample (10 μL) was added to a mixture (20 μL) of FITC-conjugated anti-GPIX, PE-conjugated anti-P-selectin, and saline or PAR4 TRAP (final concentration of 300 μM). After 15 min of incubation at room temperature, the samples were fixed with FACS lysing solution (Becton Dickinson) and analyzed in a FACS Canto II (Becton Dickinson). FITC-positive cells were considered as platelets, and the PE fluorescence was measured on 10,000 platelets and expressed as relative fluorescence units (rfu).

Statistical Analysis

Data are presented as mean ± standard error (S.E.). For 2-group comparisons, Student's *t* tests were performed, and the level of significance was set at a 2-sided 5%. SAS-based INATOX-DP system (Ina Research Inc.), SAS System Release 8.2 and 9.2 (SAS Institute Inc.), EXSAS system (CAC EXICARE Corporation), or Microsoft Excel 2010 (Microsoft Corporation) was used for statistical analyses.

Results

Plasma Concentrations of Adenine Nucleotides

The plasma concentrations of adenine nucleotides (AMP, ADP, and ATP) determined in 7-week-old SCD and control mice (n = 8 each) using LC-ESI-MS/MS are shown in Fig. 1. Adenine nucleotides in the plasma samples from SCD and control mice were quantified using the peak area of the chromatograms. The results show that the plasma concentration of ADP in 7-week-old SCD mice was 0.248 ± 0.052 μM,