Blood Cells, Molecules and Diseases 62 (2016) 13–21

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

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

# Inflammatory and oxidative stress phenotypes in transgenic sickle cell mice

Emmanuelle Charrin [a,b], Solomon Fiifi Ofori-Acquah [c,d], Elie Nader [a,b], Sarah Skinner [a,b], Philippe Connes [a,b,e], Vincent Pialoux [a,b,e], Philippe Joly [a,b,f], Cyril Martin [a,b,*]

[a] Inter-university Laboratory of Human Movement Science EA7424, Vascular biology and Red Blood Cell Team, University Claude Bernard Lyon 1, University of Lyon, Villeurbanne, France
[b] Laboratory of Excellence "GR-Ex", Paris, France
[c] Division of Hematology/Oncology, Department of Medicine, University of Pittsburgh, Pittsburgh, PA 15261, USA
[d] Center for Translational and International Hematology, Vascular Medicine Institute, University of Pittsburgh, Pittsburgh, PA 15261, USA
[e] Institut Universitaire de France, Paris, France
[f] Laboratory of Biochemistry and Molecular Biology, Hospital Edouard Herriot, Lyon, France

ARTICLE INFO

*Article history:*
Submitted 30 August 2016
Revised 18 October 2016
Accepted 26 October 2016
Available online 28 October 2016

*Keywords:*
Inflammation
Oxidative stress
Nitric oxide
Sickle cell disease

ABSTRACT

The Townes mouse model of homozygous sickle cell disease (SS) has emerged as the major experimental model for studying pathophysiological mechanisms of human sickle cell disease (SCD). We therefore investigated hematological and hemorheological parameters as well as organ-specific inflammatory and oxidative stress molecular profiles in these animals in steady state conditions. Evidences of SCD-related intravascular hemolysis, impaired red blood cell (RBC) deformability, leukocytosis and altered plasma nitric oxide byproducts (NOx) level were found in the SS mice. The SS mice have damaged, enlarged and dysfunctional spleen as attested by high AOPP levels, low SOD and GPx activities and low pro-inflammatory cytokines mRNA expression. SS mice exhibited cardiomegaly, high cardiac mRNA levels of proinflammatory markers and low cardiac GPx activity. While lungs did not display any noticeable defects, liver and kidney were particularly sensitive to oxidative stress and inflammation as suggested by high AOPP levels in both organs, elevated renal NF-κB and TNF-α, and increased hepatic VCAM-1 and IL-1β. Our data indicate a tissue-specific phenotype regarding oxidative stress and inflammation in SS mice that may help to optimize the development of novel potential drug treatments.



## 1. Introduction

Sickle cell disease (SCD) is the most common genetic disorder, affecting millions of people worldwide. This severe hemoglobinopathy results from the substitution of glutamic acid for valine at the sixth amino acid position in the beta-globin gene [1]. When deoxygenated, mutant hemoglobin (HbS) polymerizes causing the sickling of red blood cells (RBCs) [2]. Fragile and poorly deformable sickle RBCs are responsible for hemorheological alterations [3] leading to typical clinical manifestations including recurrent painful vaso-occlusive crises (VOCs) and severe intravascular hemolytic anemia. Studies of the past decades have demonstrated the key contribution of inflammation and oxidative stress in the pathophysiology of SCD [4,5]. Free heme released into the circulation as a consequence of intravascular hemolysis can intercalate into endothelial cell membranes and cause oxidative and inflammatory damage typical of SCD [6]. Repeated ischemia-reperfusion (I/R) events occurring during VOC generates reactive oxygen species (ROS) through the activation of xanthine oxidase [7]. Generation of ROS can also result from the strong activation of enzyme complexes such as NADPH oxidase and uncoupled nitric oxide synthase [8]. Thus, oxidative stress results from an imbalance between enhanced production of ROS and low levels of antioxidant defences [9,10]. Indeed, SCD patients have been shown to have reduced concentration and activity of antioxidant systems [11,12]. Moreover, the scavenging of nitric oxide (NO) by ROS inhibits the antioxidative and vasodilatory properties of NO and worsens intravascular hemolysis leading to a vicious circle [4,13]. I/R injury also activates the endothelium and increases the expression of adhesion molecules on endothelial cells [14].

Several murine models have been developed to study the complex pathophysiological mechanisms of SCD [15–19]. The model increasingly being used is the humanized Townes mouse in which the murine α- and β-globin genes have been replaced by the human α-, γ- and either $\beta^A$- or $\beta^S$-globin genes. A major advantage of the Townes model is that healthy mice expressing normal adult hemoglobin (AA mice) are generated as littermate controls for experiments with SS mice by intercrossing asymptomatic heterozygous AS (sickle cell trait, SCT) mice [5]. In addition, the Townes SS mice display numerous clinical

* Corresponding author at: Interuniversity Laboratory of Human Movement Science (LIBM EA7424), Université Claude Bernard Lyon 1, Université de Lyon, Campus La Doua, Bat Raphael Dubois, 27–29 bd du 11 Novembre 1918, 69622 Villeurbanne Cedex, France.
*E-mail address:* cyril.martin@univ-lyon1.fr (C. Martin).

http://dx.doi.org/10.1016/j.bcmd.2016.10.020
1079-9796/

manifestations consistent with the human disease such as severe anemia [20], leukocytosis [21] and multi-organ damages [19]. The Townes SS mice develop pulmonary vascular leakage and edema [22], splenic infarcts and congestion [19], hepatocellular damages [19,23] and glomerulopathy [24]. Hitherto, molecular inflammatory and oxidative stress phenotypes of major organs that are impacted by SCD have not been evaluated in this model. Thus, the present study was designed to analyze the hematological, hemorheological parameters, and inflammatory and oxidative stress molecular responses in several organs affected by SCD in the SS mice.

## 2. Material & methods

### 2.1. Animals

We have established a colony of Townes sickle mice in our laboratory originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mouse genotypes were confirmed by PCR. A total of 32 mice aged 12 to 18 weeks old were used and maintained on a 12-hour light-dark cycle with food and water ad libitum. The guidelines from the French Ministry of Agriculture for experimental procedures and the Institute for Laboratory Animal Research (National Academy of Sciences, USA) were followed and the protocol was approved by the regional animal care committee (Rhône-Alpes, France).

### 2.2. Tissue sampling

Mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/Kg, Dolethal®, Vétoquinol, Lure, France) and blood was collected by a retro-orbital venipuncture into EDTA tubes for hematological and hemorheological analysis. Mice were sacrificed by exsanguination with a 0.9% NaCl transcardial perfusion for 70 s. Heart, lungs, liver, spleen and kidneys were collected, weighted and immediately frozen in liquid nitrogen for oxidative stress, western blot and qRT-PCR analyses.

### 2.3. Hematology

An ABX Micros 60 automat (Horiba, Montpellier, France) was used for the following hematological measurements: hematocrit (Hct), red blood cell (RBC) count, hemoglobin concentration, mean corpuscular volume (MCV), RBC distribution width (RDW), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), total white blood cell (WBC), lymphocyte, monocyte, granulocyte and platelet count and mean platelet volume (MPV). The percentage of reticulocytes was assessed under a light microscope in a blind fashion on smears stained with brilliant cresyl blue (860867, Sigma-Aldrich, St-Louis, MO, USA) by two independent investigators (BX43 Microscope, Olympus, Tokyo, Japan).

### 2.4. Hemorheology

RBC deformability was determined at 37 °C at two shear stresses (3 and 30 Pa) by laser diffraction analysis (ecktacytometry), using the Laser-assisted Optical Rotational Cell Analyzer (LORCA MaxSis, RR Mechatronics, Hoorn, The Netherlands). Briefly, 7 μL of oxygenated blood suspension was mixed with 1 mL polyvinylpyrrolidone (PVP; viscosity = 30 cP, RR Mechatronics, Hoorn, The Netherlands) and sheared into the glass Couette system. The diffraction pattern was analyzed by the computer and an elongation index was calculated according to recent recommendations [25]. An increase of the elongation index indicates increased RBC deformability. Whole blood viscosity was determined with a cone plate viscometer (Brookfield DVII+, with CPE40 spindle) at a shear rate of 225 $s^{-1}$.

### 2.5. qRT-PCR for antioxidant enzymes mRNA expression

Total mRNA from organs was isolated using RNeasy mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. 500 ng per sample of total mRNA were reverse transcribed to cDNA with the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The reactions were incubated in a thermal cycler for 10 min at 25 °c, 2 h at 37 °c, 5 min at 85 °c and then held at 4 °c. Real-time qPCR analysis was performed on a StepOne Realtime PCR system (Applied Biosystems, Foster City, CA, USA). All experiments were done in triplicate with TaqMan fast advanced master mix (Applied Biosystems, Foster City, CA, USA). Expression level was calculated by the ddCt method and normalized to ribosomal 18S RNA [26]. The TaqMan probes used were: *Heme oxygenase-1* (Hmox-1; Mm00516005_m1), *Nadph quinone oxidoreductase-1* (Nqo-1; Mm01253561_m1), *Superoxide dismutase-1* (Sod-1; Mm01344233_g1).

### 2.6. qRT-PCR for cytokines mRNA expression

Total mRNA from organs was isolated using Trizol reagent (Tri Reagent LS, Euromedex, Souffelweyersheim, France) according to the manufacturer instructions, purified with DNase I (EN0525, ThermoFisher scientific, Waltham, MA, USA) and concentrated at 80 ng/μL. 1000 ng per sample of total mRNA were reverse transcribed to cDNA with the reverse transcriptase RNase Hminus (Promega, Madison, WI, USA) using oligo (T)15 (Eurogentec, Seraing, Belgium). RT calibration was done in the presence of 80 pg of a synthetic external and non-homologous poly(A) Standard RNA (SmRNA) used to normalise the reverse transcription of mRNAs of biological samples (Morales and Bezin, patent WO2004.092414). Real-time qPCR analysis was performed on a Rotor-Gene Q system (Qiagen, Venlo, Netherlands) by using the Rotor-Gene SYBR® green PCR kit (Qiagen, Venlo, Netherlands). The thermal profiles consisted of 15 min at 95 °C for denaturing followed by 45 cycles of amplifications (15 s at 94 °C for denaturation, 30 s at 58 °C for annealing and 6 s at 72 °C for extension). Results obtained for the targeted mRNAs were normalized against the SmRNA. The primer pairs used were: *Vascular cell adhesion molecule-1* (VCAM-1; NM_011693.2); *Interleukine-1β* (IL-1β; NM_008361.3); *Interleukine-6* (IL-6; M24221.1); *Tumor necrosis factor-α* (TNF-α; M13049.1); *Endothelial nitric oxide synthase* (eNOS; NM_008713.4).

### 2.7. Oxidative stress and antioxidant assessment

Heart, liver, spleen and kidney were homogenized (10%, w/v) in PBS 1X + EDTA 0.5 mM in ice. After centrifugation at 12,000*g* for 10 min at 4 °C, the supernatant was collected for oxidative stress markers measurements. Homogenate aliquots were stored at −80 °C. Protein concentrations were determined using the BCA protein assays Kit (Novagen, Darmstadt, Germany) in accordance to the manufacturer's instructions. All the products used for oxidative stress measurements were purchased from Sigma-Aldrich (St-Louis, MO, USA). Spectrophotometric measurements were performed on TECAN Infinite 2000 plate reader (Männedorf, Switzerland). Results were standardized per mg of total protein.

Advanced oxidation protein products (AOPP) were determined using the method developed by Witko-Sarsat et al. [27] as previously described [28]. AOPP were calibrated with a chloramine-T solution that absorbs at 340 nm in the presence of potassium iodide. The absorbance of the reaction was immediately read at 340 nm on the microplate reader against a blank containing 200 μL of PBS. AOPP activity was expressed as micromoles per liter of chloramine-T equivalents.

Glutathione peroxidase (GPx) activity was determined by the modified method of Paglia and Valentine [29]. GPx activity represents the rate of NADPH elimination in $NADP^+$ after addition of glutathione