Blood Cells, Molecules and Diseases 55 (2015) 363–372

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

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

Rapamycin increases fetal hemoglobin and ameliorates the nociception phenotype in sickle cell mice

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ARTICLE INFO

Article history:
Submitted 19 April 2015
Revised 3 August 2015
Accepted 3 August 2015
Available online 5 August 2015

Editor: Mohandas Narla

Keywords:
Sickle cell disease
Rapamycin
mTOR
Pain
Nociception
Sine wave
Fetal hemoglobin

ABSTRACT

Fetal hemoglobin-inducing therapies are disease-modifying and ameliorate the pain phenotype in sickle cell disease (SCD). Rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, increases HbF in erythroid precursor cells in vitro. We hypothesized that rapamycin would increase HbF levels and improve nociception phenotype in SCD mice. We used sine-wave electrical stimulation to examine nocifensive phenotype and evaluate myelinated [2000 Hz (Aβ-fiber) and 250 Hz (Aδ-fiber)] and unmyelinated (5 Hz C-fibers)] sensory fiber function. Rapamycin significantly increased γ-globin mRNA and HbF levels [+2.3% (0.7, 3.9), mean increase (95% confidence interval, CI), $p = 0.006$]. In homozygous (sickling) mice, long- (16 weeks), but not short-term (6 weeks), rapamycin treatment increased 2000 Hz and 250 Hz current thresholds in a pattern that varied according to sex. In male, but not female mice, rapamycin (compared with vehicle) was associated with increases in 2000 Hz [21 Units (7, 35), mean difference (95% CI), $p = 0.009$ for sex * treatment interaction] and 250 Hz [9 Units (1, 16), $p = 0.01$] current thresholds. In rapamycin-treated homozygotes, HbF levels directly correlated with myelinated [2000 Hz(Aβ-fiber, $r = 0.58$, $p = 0.01$) and 250 Hz(Aδ-fiber, $r = 0.6$, $p = 0.01$)] but not unmyelinated sensory fiber current thresholds. These findings suggest that in SCD mice, rapamycin increases HbF and modulates current thresholds of myelinated fibers. Therefore, mTOR signaling might be implicated in the pathobiology of SCD.

1. Introduction

Therapies that increase fetal hemoglobin are the only known disease-modifying treatments for patients with sickle cell disease (SCD). One such therapy, hydroxyurea, a ribonucleotide reductase inhibitor, has been show to decrease pain frequency, number of vasoocclusive crises, analgesic use, as well as hospital admissions in large multicenter randomized clinical trials in SCD patients [1–6]. While researchers believe that the benefits of hydroxyurea in SCD are related to its fetal hemoglobin-inducing effects, hydroxyurea has also been shown to improve erythrocyte morphology, decrease adhesion molecule expression, and decrease hemolysis [7–9]. Consequently, hydroxyurea is often prescribed for SCD patients who have frequent pain crises and/or neuropathic pain [8,10,11]. However, because some patients fail to respond to or do not tolerate hydroxyurea, alternative fetal hemoglobin-inducing strategies are needed.

The serine/threonine protein kinase mammalian target of rapamycin (mTOR) is pivotal for the process of cell growth and division as it senses and couples energy, nutrient availability, and protein production at the translational level [12]. During erythropoiesis, the mTOR pathway is a critical regulator of growth and proliferation of red blood cells both in vitro and in vivo [13–15]. Inhibition of the mTOR pathway with rapamycin has significant effects on erythropoiesis and some of those effects are relevant to SCD. For example, in erythroid precursor cells from normal subjects and patients with β-thalassemia, the inhibition of the mTOR pathway with rapamycin increases the expression of fetal hemoglobin, an effect that is highly relevant to SCD [16,17]. In vivo, rapamycin treatment also increases red blood cell counts and hemoglobin levels in β-thalassemic mice [13]. Therefore, given these fetal hemoglobin-inducing properties in vitro in erythroid precursor cells and its effects in vivo in β-thalassemic mice, it is conceivable that rapamycin has a role in erythropoiesis in SCD.

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http://dx.doi.org/10.1016/j.bcmd.2015.08.001
1079-9796/

Some humanized SCD mouse strains express human fetal hemoglobin and allow for the evaluation of drugs that have fetal hemoglobin-inducing properties [18,19]. In addition, these mice recapitulate many of the phenotypes observed in SCD including altered nociception characterized mechanical allodynia [20], thermal and mechanical hyperalgesia, and sensitization of sensory nerve fibers [19–24]. Therefore, the humanized SCD mouse provides an ideal model for the evaluation of the effect of fetal hemoglobin-inducing therapies on the nociception phenotype in these mice. In the present investigation, we examined the effect of rapamycin on humanized SCD mice and hypothesized that rapamycin would increase fetal hemoglobin levels and ameliorate the nocifensive behavior phenotype in sickle cell mice.

2. Methods

2.1. Animals

The Children's National Health System Institutional Animal Care and Use Committee approved the investigational protocol. The animals enrolled in the study (referred to as "Townes mice", B6;129-*Hba*$^{tm1(HBA)Tow}$ *Hbb*$^{tm2(HBG1,HBB^*)Tow}$/*Hbb*$^{tm3(HBG1,HBB)Tow}$/J) were purchased from the Jackson Laboratory (stock number 013071), Bar Harbor, ME and bred in our animal facility. These animals do not express mouse hemoglobin and instead express human hemoglobin genes as follows [25,26]. Homozygous (sickling) Townes mice (hα/hα::β^S/β^S) carry mutations containing the human globin α-globin (*Hba*$^{tm1(HBA)Tow}$, hα) and the human ^{A}γ-globin and human sickle hemoglobin beta genes (*Hbb*$^{tm2(HBG1,HBB^*)Tow}$, β^S). Heterozygous (non-sickling) Townes mice (hα/hα::β^A/β^S) carry the (hα) mutation, one copy of the human β^S (as above) and one copy of the human wild-type hemoglobin beta (*Hbb*$^{tm3(HBG1,HBB)Tow}$, β^A) genes. Homozygous Townes mice have several hematologic defects seen in humans with sickle cell anemia (anemia, reticulocytosis, leukocytosis, sickling) as well as liver and kidney diseases [25,26]. Heterozygous Townes mice have significantly less hematologic abnormalities akin to humans with sickle cell trait [25,26]. Mice were housed in ventilated cages in a temperature-controlled environment (21 °C), with standard 12-h light–dark cycle.

2.2. Rapamycin treatment

In this study, we followed guidelines to optimize the predictive value of preclinical research [27]. As rapamycin effects have been shown to vary according to treatment duration [28], we first treated one animal cohort (heterozygous and homozygous) for 16 weeks and then another for six weeks with rapamycin or vehicle.

Rapamycin (LC Labs, Woburn, MA) at a ratio of 14 mg/kg of mouse chow was microencapsulated with the enteric coating material Eudragit S100 (Röhm Pharma, Germany) by Southwest Research Institute (San Antonio, TX), using a spinning disk atomization coating process as previously described [29,30]. During the treatment, mice were given free access to water and to mouse chow containing either microencapsulated rapamycin or control diet (microencapsulating material, but no rapamycin) [29]. Assuming that a mouse consumes approximately 5 g of mouse chow daily, microencapsulated rapamycin-fed mice consumed approximately 2.24 mg of rapamycin/kg/day [29]. This administration regimen has been shown to yield extensive rapamycin tissue distribution in mice, yield stable brain levels, increase cerebral blood flow and vascular density, and to extend mouse lifespan [29,30].

2.3. Fetal hemoglobin levels

We used capillary zone electrophoresis using the Hemoglobin Assay program on MINICAP™ (Sebia, Norcross, GA) to quantitate and identify the different human hemoglobin fractions per manufacturer's recommendations. Mice red blood cell hemolysates from the short and long rapamycin trials were prepared and injected by aspiration at the anionic end of the capillary. High voltage protein separation was then performed and followed by direct detection of the hemoglobins at 415 nm. Fetal hemoglobin levels are expressed as percentage of total hemoglobin.

2.4. RNA preparation and real time RT-PCR

RNA was isolated from the spleen, a site of stress hematopoiesis in SCD mice, as well as from the bone marrow using the RNeasy mini kit (Qiagen, Valencia, CA), according to the manufacturer's protocol and its concentration was determined using NanoDrop (NanoDrop, Wilmington, DE). Total reaction mixture volume was 10 μL. No template controls were used for each probe to control for contamination. The PCR conditions included a cDNA synthesis step for 30 min at 48 °C, followed by a polymerase activation step at 95 °C for 10 min, and then by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The reactions were run on an ABI PRISM HT-7900 Sequence Detector (Applied Biosystems, Foster City, CA). Amplification reactions were performed in a mixture containing 10 μL of 2× TaqMan One Step PCR Master Mix, 0.5 μL of 40× Multiscribe and RNase Inhibitor Mix (all from Applied Biosystems, Foster City, CA) and 2 μL of target RNA (50 ng/μL). Each reaction contained either 1 μL of 20× Assays-on-Demand Gene Expression probe for murine *Klf-1*, *Bcl11a*, β-actin or 1 μL of custom-made probe–primer mixture for human HbF (all from Applied Biosystems). Human HbF primers had the following sequences: the forward primer 5′ CAACCCCAAAGTCAAG GCAC, reverse primer 5′ GATTGCCAAAACGGTCACCA and a probe 5'ACTTCCTTGGGAGATGCCACAAAGCAC. Note that these primers detect both *Homo sapiens* gamma A (HBG1) and gamma G (HBG2) hemoglobin mRNAs.

2.5. Hematological, biochemical, and histological parameters

Blood was collected from anesthetized animals via cardiac puncture into heparin-coated syringes and left for 10 min at room temperature. Complete blood cell counts were obtained on a Hemavet blood counter (Drew Scientific, Dallas, TX). Blood then was centrifuged (1500 *g* for 10 min at 4 °C), plasma separated and frozen at −80 °C. Total bilirubin was measured using QuantiChrom™ Bilirubin Assay Kit (BioAssay Systems, Hayward, CA), according to the manufacturer's instructions. Plasma free hemoglobin was assayed using a colorimetric assay (Catachem, Oxford, CT). Assay conditions were optimized for 96 well plates by using 2 μL of plasma and reducing assay reagents proportionally per manufacturer's instructions. Each sample was assayed in triplicate and an average was obtained.

In a third cohort of mice treated with rapamycin (N = 5) or vehicle (N = 5), we examined bone-marrow histology. Formalin-fixed bone marrow sections (4 μm) of the tibia were stained with hematoxylin and eosin and myeloid to erythroid ratio (M:E) was analyzed by an investigator (IM) unaware of treatment groups.

2.6. Nocifensive behavior studies

In both long and short trials, current vocalization threshold measurements were obtained at baseline (before) and after rapamycin or vehicle treatments. In the long rapamycin trial, nocifensive behavior measurements (current vocalization threshold only) were also obtained after 8 weeks of treatment. Thermosensory response measurements were obtained before and after the short, but not the long, rapamycin trial.

2.7. Current vocalization threshold

We examined sensory nerve fiber function using sine wave electrical stimulation at three different frequencies: 2000, 250, and 5 Hz, which preferentially stimulate Aβ, Aδ, and C fibers respectively [31–39]. Briefly, electrical stimuli generated by a neurostimulator (Neurotron, Inc.,