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Isocitrate treatment of acute anemia of inflammation in a mouse model

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ABSTRACT

Acute and severe anemia of inflammation (AI) is a common complication of various clinical syndromes, including fulminant infections, critical illness with multiorgan failure, and exacerbations of autoimmune diseases. Building on recent data showing beneficial results with isocitrate treatment for chronic low-grade AI in a rat model, we used a mouse model of acute and severe AI induced by intraperitoneal heat-killed Brucella abortus to determine if isocitrate would be effective in this more stringent application. Inflamed mice treated with isocitrate developed an early but transient improvement in hemoglobin compared to solvent-treated controls, with a robust improvement on day 7, and only a trend towards improvement by day 14. Reticulocyte counts were increased in treated mice transiently, with no significant difference by day 21. Serum erythropoietin (EPO) levels were similar in treated versus control mice, indicating that isocitrate increased sensitivity to EPO. Serum and tissue iron levels showed no significant differences between the treated and control mice, ruling out improved iron availability as the cause of the increased response to endogenous EPO. Compared to the milder rat model, much higher doses of isocitrate were required for a relatively modest benefit.

1. Introduction

Anemia of inflammation (AI) is a feature of a broad spectrum of inflammatory disorders, including connective tissue diseases, infections, certain malignancies, and chronic kidney disease [1]. The phenotype of AI is typically a normocytic normochromic anemia with a shortened erythrocyte lifespan and suppressed erythropoiesis despite adequate levels of circulating erythropoietin (EPO) [2]. Perhaps the most characteristic feature of AI is a dysregulation of systemic iron homeostasis characterized by hypoferremia despite intact iron stores [1], resulting in decreased availability of iron for hemoglobin synthesis and erythrocyte production. Evolutionarily, this inflammatory response limited iron availability to microbes during infections.

The principal regulator of iron homeostasis is hepcidin, a 25-amino acid peptide hormone produced primarily by hepatocytes [3]. Hepcidin acts by binding to ferroportin, the sole known cellular iron exporter that is expressed on the surface of macrophages, hepatocytes, and the basolateral membranes of enterocytes. Hepcidin binding causes ferroportin endocytosis and degradation [4], inhibiting enteral iron absorption and the efflux of cellular iron necessary for erythropoiesis. Excessive hepcidin production causes hypoferremia and iron sequestration in macrophages, as seen in transgenic mice with hepcidin overexpression [5] and in the human genetic syndrome iron-refractory iron-deficiency anemia (IRIDA) that occurs due to mutations in matriptase-2/TMPRSS6 [6]. During inflammation or infection, hepcidin expression is strongly stimulated, largely by IL-6 [7] via the JAK-STAT pathway [8–10].

Our group recently published a detailed characterization of a mouse model of acute and severe inflammation that served as the platform for the current studies [11]. With a single intraperitoneal injection of Brucella abortus (BA), mice develop a severe anemia with a hemoglobin nadir at day 14, iron restriction despite increased tissue iron stores, erythropoietic suppression, and a shortened erythrocyte lifespan. Hepcidin ablation causes a partial correction of the resulting anemia, accompanied by an accelerated recovery [11]. In short, this model displays all the major characteristics seen in AI, and is an effective vehicle for testing any potential interventions for acute and severe AI.

Recent in vitro and murine studies have shown beneficial results with isocitrate treatment for anemia of inflammation. In particular, Richardson et al. showed a significant and persistent attenuation of anemia in a rat arthritis model of moderate chronic AI with a short course of isocitrate treatment [12]. Building on these promising data, the current study uses the BA mouse model to investigate the potential therapeutic use of isocitrate in the context of a very acute and severe

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inflammation. We examined the effects of isocitrate treatment on hemoglobin levels and erythropoiesis, as well as the mechanistic contributions of EPO production, changes in iron status, and inflammation.

2. Methods

2.1. Animal models

All animal studies were approved by the Animal Research Committee at University of California, Los Angeles (UCLA). Only male mice were used in the study to avoid the effects of gender-related differences in iron parameters and hepcidin [13]. C57BL/6 J mice were obtained from Charles River Laboratories (Wilmington, MA) or The Jackson Laboratories (Bar Harbor, ME). Wild-type (WT) mice were maintained on a standard chow (approx. 270 ppm iron, Harlan Teklad, Indianopolis, IN) until 6 weeks of age and they were then switched to an iron-adequate diet (50 ppm iron, Harlan Teklad) for 14–15 days before injection of BA. The same iron-adequate diet was continued through the remainder of the experiment. The dietary conditioning was applied because the high iron content of standard chow maximally stimulates hepcidin expression and renders it unresponsive to inflammatory stimuli [7], and because dietary iron absorption in humans accounts for ~5–10% of the daily iron fluxes but as much as ~50% in mice fed standard chow [14]. High dietary iron absorption in mice may obscure the contribution of iron recycling by macrophages [15] and lead to progressive iron loading. Reducing the dietary iron content of mouse chow was designed to model iron fluxes of human homeostasis.

To induce AI, animals were injected intraperitoneally (IP) with 5×10^8 particles/mouse of heat-killed B. abortus (BA; lots 5–1101 and 5–1304; US Department of Agriculture, Animal and Plant Health Inspection Service, National Veterinary Services Laboratories) as previously described [16]. Daily IP injections of 100 mg/kg/d of trisodium isocitrate (Sigma-Aldrich; Saint Louis, MO) dissolved in 200 μL 0.9% saline, or equivalent volumes of 0.9% saline solution, were initiated 3 days prior to B. abortus injection and continued for a total of 9 days). Mice were euthanized at 3 time points (7 d, 14 d, 21 d) and blood, liver, and spleen were collected at necropsy. See Supplementary Fig. 1 for the experimental timeline diagram. For each time point, 8 isocitrate-treated and 8 saline-treated mice came from the same cohort and were processed simultaneously to minimize cohort-to-cohort variability. A second set of 8 isocitrate-injected and 8 saline-injected mice was analyzed at 7 d to confirm the significant hematologic differences seen at that time point. A final set of 16 mice was analyzed in order to evaluate the effect of isocitrate in uninflamed mice. Saline-injected mice were treated with either isocitrate or saline, then analyzed at day 7.

2.2. Hematologic studies

Complete blood counts were obtained with a HemaVet blood analyzer (Drew Scientific; Waterbury, CT). To assess iron-restricted erythropoiesis, zinc protoporphyrin (ZPP) was measured using a hematofluorometer (AVIV; Lakewood, NJ).

Reticulocytes were counted by flow cytometry. Blood (5 μl) was added to 1 ml of thiazole orange in phosphate buffered saline with 0.1% sodium azide (PBS-azide, BD Bioscience; San Jose, CA) and incubated at room temperature for 1–3 h. As an unstained control, blood was added to PBS-azide without thiazole orange. The percentage of red-fluorescent reticulocytes (Retic %) was measured by flow cytometry at the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research Flow Cytometry Core Facility that is supported by National Institutes of Health awards CA-16,042 and AI-28,697, and by the JCCC, the UCLA AIDS Institute, and the David Geffen School of Medicine at UCLA. Unstained controls were used to establish a gate to exclude background fluorescence. The results are expressed as the reticulocyte count (M/μL) = RBC (M/μL) × Retic %. Spleen weight measurements were also obtained from a large subset of the mice (7–12 mice per treatment group per time point) as a marker of extramedullary erythropoiesis..

2.3. Measurement of iron parameters, serum EPO, and serum lactate dehydrogenase (LDH)

Serum iron and liver and spleen non-heme iron concentrations were determined by a colorimetric assay for iron quantification (Sekisui Diagnostics; Lexington, MA) as previously described [14]. Serum EPO was measured from a subset of the mice (7–8 mice per treatment group per time point) using a solid-phase enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D; Minneapolis, MN). Serum LDH was measured from a subset of the mice (4–6 mice per treatment group per time point) at our Diagnostic Lab Services core facilities (Division of Laboratory Animal Medicine; University of California, Los Angeles).

2.4. RNA isolation and real-time quantitative PCR

Total liver RNA was isolated from a subset of the mice (7–8 mice per treatment group per time point), and analyzed by real-time RT-PCR as described previously [17]. Primers are listed in Supplementary Table 1.

2.5. Statistics

SigmaStat 12.5 was used for all statistical analyses (Systat Software; Point Richmond, CA). Normally distributed data were compared using Student's t-test. We used the resulting one-tailed p values to evaluate the data, as our experiments were specifically designed to test whether we could replicate the beneficial effects of isocitrate seen in the Goldfarb study [12] in a mouse model of acute inflammation. Measurements that were not normally distributed were compared by the nonparametric Mann–Whitney rank sum test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Isocitrate treatment improves anemia and increases reticulocytosis in a mouse model of acute and severe inflammation

We employed our previously characterized BA mouse model of AI in order to investigate the effects of isocitrate on the anemia of acute and severe inflammation [11]. For the current studies, groups of BA-injected mice were treated with multiple injections of isocitrate or saline, then sacrificed and analyzed at various time points. A pilot trial was performed using 3 injections of isocitrate 400 mg/kg/d, double the weight-based dose used in the previous Richardson et al. study that showed anemia attenuation with isocitrate in a rat model of chronic inflammation [12]. As this pilot trial yielded no increase in hemoglobin (Hgb) or reticulocyte counts [data not shown], we proceeded with a more intensive treatment regimen using 9 daily injections of isocitrate 1000 mg/kg/d that began 3 days prior to BA injection.

Inflamed mice treated with this regimen of isocitrate developed a milder anemia at 7 d than saline-treated mice (Fig. 1A, mean isocitrate Hgb 10.9 g/dL vs. saline Hgb 7.2 g/dL; $p < 0.001$). This benefit was transient and no longer significant by 14 d, although there was still a trend towards milder anemia at 14 d (median isocitrate Hgb 8.6 g/dL vs. saline Hgb 7.0 g/dL; $p = 0.094$). After the expected Hgb nadir of both groups at 14 d, both treated and untreated groups had partially recovered their Hgb levels by 21 d (mean isocitrate Hgb 11.4 g/dL vs. saline Hgb 11.0 g/dL). In order to explore the possibility that isocitrate alone could affect Hgb without inflammation, we repeated the 7 d experiment using mice that were not injected with BA. These saline-injected mice were then treated with either isocitrate or saline and analyzed at 7 d. The treatment groups had no difference in Hgb levels (Supplementary Fig. 2),