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Low level arsenite exposures suppress the development of bone marrow erythroid progenitors and result in anemia in adult male mice

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ABSTRACT

Epidemiological studies report an association between chronic arsenic (As) exposure and anemia in men, and women who are predisposed to anemia. The purpose of these studies was to determine whether a 60 d drinking water exposure of adult male C57BL/6J mice to 0, 100, and 500 ppb arsenite (As^{+3}) results in anemia due to alterations in erythroid progenitor cell development in the bone marrow. Exposure to 500 ppb As^{+3} for 60 d resulted in a reduction of mean corpuscular hemoglobin (MCH) levels, but did not significantly alter red blood cell (RBC) counts, hemoglobin (Hgb) levels, mean corpuscular Hgb concentrations (MCHC), or mean corpuscular volumes (MCV). Attenuation of burst-forming unit-erythroid (BFU-E) colony formation was observed in bone marrow cells of mice exposed to 500 ppb As^{+3}. The differentiation of late-stage bone marrow erythroblasts as defined by CD71 and Ter119 surface marker expression was reduced with the 500 ppb As^{+3} exposure. Mice exposed to 500 ppb As^{+3} also had elevated serum levels of erythropoietin (EPO). Collectively, these results show that exposure to low levels of As^{+3} attenuate the development of early BFU-E cells and reduce the differentiation of late-stage erythroblasts. This suppression of bone marrow erythropoiesis may be a contributing factor to the mild hypochromic anemia observed in 500 ppb As^{+3} exposed mice.

1. Introduction

As is a widespread environmental toxicant and common contaminant in food and drinking water (WHO, 2011; Naujokas et al., 2013). Many people are chronically exposed to levels of As in their drinking water that exceed the World Health Organization and United States Environmental Protection Agency maximum contaminant level of 10 ppb (U.S. EPA 2012; WHO, 2011). As occurs in the environment in organic and inorganic forms with multiple valence states (*i.e.* +3 or +5) that have differential toxicological profiles (Petrick et al., 2001; Styblo et al., 2000; Szymańska-Chabowska et al., 2002). As^{+3} is commonly found in drinking water and is the most toxic inorganic form of As (Styblo et al., 2000; Naujokas et al., 2013). Exposure to elevated levels of As^{+3} has been documented to exert a multitude of detrimental health outcomes, including cancers, cardiovascular diseases, immunosuppression, and anemia (Hughes, 2002; Heck et al., 2008; Naujokas et al., 2013; Ferrario et al., 2016).

Anemia is classified as a decrease in the number of RBCs and/or reduced Hgb levels in circulating RBCs (WHO, 2015). Multiple epidemiological studies report an association between chronic As exposure and anemia (Heck et al., 2008; Surdu et al., 2015; Kile et al., 2016). Heck et al. (2008), found that low Hgb levels (< 10 g/dL) were negatively associated with urinary As concentrations (> 200 ppb) in men and women living in Bangladesh. Pregnant women exposed to elevated levels of As in their drinking water are particularly susceptible to developing anemia (Hopenhayn et al., 2006; Surdu et al., 2015). Findings from these studies emphasize the need to develop a clear understanding of the relationship between environmentally relevant As^{+3} exposures and anemia.

The bone marrow is very sensitive to As-induced toxicity (Szymańska-Chabowska et al., 2002; Ezeh et al., 2014, 2016; Xu et al., 2016) and is the major site of erythropoiesis in adult humans and mice (Tsiftsoglou et al., 2009; Dzierzak and Philipsen, 2013). Erythropoiesis is regulated by EPO released from the kidney in response to hypoxic conditions in the body (Hattangadi et al., 2011). Increased EPO levels stimulate the proliferation and differentiation of early erythroid progenitor cells in the bone marrow (Hattangadi et al., 2011). The first stage of erythroblast differentiation is BFU-E, which respond to increased EPO and other growth factors (*e.g.*, SCF, IL-3, IL-6) to proliferate and mature to the highly EPO responsive colony-forming

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unit-erythroid (CFU-E) stage (Hattangadi et al., 2011). At the CFU-E stage, Hgb production is initiated, and the cells undergo four additional stages of differentiation (*i.e.* proerythroblast, basophilc, polychromatophilic, and orthochromatophilic), prior to enucleation and release from the bone marrow into circulation (Migliaccio, 2010; Elliott and Sinclair, 2012).

The purpose of this study was to determine whether a 60 d drinking water exposure of adult male C57BL/6J mice to environmentally relevant levels of As^{+3} (0, 100, and 500 ppb) results in anemia. As a potential target for As^{+3}-induced toxicity with relevance to anemia, we evaluated the colony forming ability and differentiation of early erythroid progenitor cells in the bone marrow.

2. Materials and methods

2.1. Chemicals and reagents

Sodium meta-arsenite (CAS 774-46-5, Cat. No. S7400), Dulbecco's phosphate buffered saline w/o Ca^{+2} or Mg^{+2} ($DPBS^{-}$), and Isocove's Modified Dulbecco's Medium were purchased from Sigma-Aldrich (St. Louis, MO). Hanks Balanced Salt Solution (HBSS) was purchased from Lonza (Walkersville, MD). Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). Penicillin/Streptomycin 10,000 (mg/ml)/10,000 (U/ml) and 200 mM L-Glutamine was purchased from Life Technologies (Grand Island, NY). Serum-free methylcellulose-based medium containing EPO for culture of mouse erythroid cells (Cat. No. SF M3436) was purchased from STEMCELL Technologies (Cambridge, MA). FITC rat anti-mouse Ter119 clone Ter119 (Cat. No. 557915) and PE rat anti-mouse CD71 clone C2 (Cat. No. 553267) antibodies were purchased from BD Biosciences (San Jose, CA). Cellometer acridine orange/propidium iodide (AO/PI) staining solution in PBS (Cat. No. CS2-0106-5ML) was purchased from Nexcelom Bioscience (Manchester, UK). Mouse EPO Quantikine® ELISA kit (Cat. No. MEP00B) was purchased from R & D Systems (Minneapolis, MN).

2.2. Mouse drinking water exposures

All experiments were performed in accordance with protocols approved by the Institutional Animal Use and Care Committee at the University of New Mexico Health Sciences Center. Male C57BL/6J mice were purchased at 8 weeks of age from Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate in our animal facility for one week prior to the onset of experiments. Mice were maintained on a 12:12 reverse light:dark cycle and were fed 2020X Teklad global soy protein-free rodent diet (Envigo, Indianapolis, IN) throughout the experiment. Mice were housed 2–3 individuals per cage and exposed to 0 (control), 100, or 500 ppb As^{+3} in their drinking water for 60 d (n = 5 mice/group). As^{+3} doses were prepared fresh weekly by weighing each water bag and determining the appropriate volume of stock As^{+3} to add into each bag to yield 100 or 500 ppb As^{+3}. Water bags were collected and weighed at the end of each week and the change in weight was used to estimate water consumption by mice in each cage.

2.3. Primary bone marrow cell isolation

Bone marrow cells were isolated as previously described (Ezeh et al., 2014). Both femurs from each mouse were harvested and placed into cold HBSS. Femurs were then transferred into a 60 mm dish with HBSS to trim excess tissue from the bone. Trimmed femurs were placed into a 60 mm dish containing 5 mL cold colony-forming unit (CFU) medium (Isocove's Modified Dulbecco's Medium supplemented with 2% heat inactivated FBS, 20 mM L-glutamine, and 100 mg/ml streptomycin and 100 units/ml penicillin) and the ends of each femur were carefully cut to reveal the interior marrow shaft. Bone marrow cells were flushed from each femur by passing approximately 6–9 mL of CFU medium through the marrow shaft using a 1cc syringe and 25-G needle. The cell suspensions were then transferred to a 15 mL centrifuge tube, centrifuged at 200 *g* for 10 min, and resuspended in 5 mL of CFU medium. Cell viabilities and concentrations were determined using AO/PI staining and a Nexcelom Cellometer® Auto 2000 (Nexcelom Bioscience, Manchester, UK).

2.4. Blood collection, serum preparation, and hematological analysis

Whole blood and blood for serum preparation was collected at the time of sacrifice by cardiac puncture into EDTA coated 250 µL tubes or 1.5 mL microcentrifuge tubes, respectively. Hematological analysis of whole blood was performed using an Abaxis VetScan HM5 hematology analyzer (Abaxis, Union City, CA). For serum preparation, blood was clotted for 2 h at room temperature (RT). Clotted blood was centrifuged at 2000 *g* for 30 mins and serum was carefully removed and transferred to a clean 1.5 mL microcentrifuge tube for storage at −80 °C.

2.5. BFU-E assay

Mouse BFU-E assays were setup following manufacturer's instructions described in version 3.4.0 of STEMCELL Technologies Technical Manual for Mouse Colony-Forming Unit Assays using MethoCult™. Bone marrow cells from both femurs of each mouse were pooled and resuspended to 1×10^6 cells/mL in CFU medium. 400 µL of the 1×10^6 cells/mL solution (4×10^5 cells) was transferred into 4 mL SF M3436 methylcellulose-based medium containing EPO to promote BFU-E development and expansion. Samples were then mixed thoroughly by vortexing and held for approximately 10 mins to allow bubbles to dissipate. 1 mL (1×10^5 cells) of each sample solution was then transferred in triplicate to treated 35 mm culture dishes (STEMCELL Technologies, Cambridge, MA) using a 5cc syringe and a 16-G blunt-end needle. Each plate was then gently rocked back and forth to evenly distribute the media across the surface of the dish. Two culture dishes and one uncovered 35 mm dish containing 3 mL of sterile water were placed into a covered 100 mm dish and incubated at 37 °C in humidified incubator with 5% CO_2 for 14 d. After 14 d of culture, BFU-E colonies containing at least 30 cells were counted based on morphology using a dissecting microscope. Colony counts are reported as the number of BFU-E colonies per million bone marrow cells.

2.6. Flow cytometry

Bone marrow erythroblast subsets were evaluated based on CD71 and Ter119 surface marker expression. 1×10^6 bone marrow cells from each mouse were transferred to 12 × 75 mm tubes and stained in 100 µL of flow stain/wash buffer ($DPBS^{-}$ with 2% heat inactivated FBS and 0.09% sodium azide) with 0.5 µg of rat anti-mouse CD71-PE and rat anti-mouse Ter119-FITC monoclonal antibodies at RT for 30 mins. Samples were then washed twice with flow stain/wash buffer. After the final wash, samples were resuspended in 0.5 mL flow stain/wash buffer and analyzed using an Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA).

2.7. Mouse EPO ELISA

Serum EPO levels were measured using the Mouse EPO Quantikine® ELISA kit according to manufacturer's instructions. Briefly, serum samples and EPO standards were diluted two-fold and 50 µL of each sample was added in duplicate to the appropriate wells of a microplate pre-coated with an EPO specific monoclonal antibody. The plate was then covered and incubated at RT for 2 h on a microplate shaker. Following incubation, the plate was washed five times. After the last wash, the plate was emptied and 100 µL of mouse EPO monoclonal antibody conjugated to horse radish peroxidase was added to each well and incubated at RT for 2 h on a microplate shaker. The plate was washed again and 100 µL of substrate solution (hydrogen peroxide and

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