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Antimalarial drug artemisinin depletes erythrocytes by activating apoptotic pathways in zebrafish

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Despite its extraordinary efficacy, administration of the major antimalarial drug artemisinin leads to anemia, and the underlying cellular and molecular mechanisms are not well understood. Here, we report the effects of artemisinin on erythroid development and apoptosis in zebrafish and human cells. By performing a small-molecule screen with zebrafish embryos, we found that artemisinin treatment depleted red blood cells and slightly decreased definitive hematopoietic stem cells, but had no effect on primitive hematopoietic progenitors. RNA-Seq revealed that artemisinin suppressed a cluster of genes in the heme biosynthesis and globin synthesis pathways. Furthermore, artemisinin induced apoptosis in erythrocytes in zebrafish embryos, as assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling assay, and preferentially acted on differentiated erythrocytes by elevating caspase 8 and caspase 9 activity in differentiated human K562 cells. Consistently, artemisinin suppressed the ectopic expression of erythroid genes in jak2aV581F-injected embryos, a zebrafish model for human polycythemia vera in which the bone marrow makes too many red blood cells. Taken together, our data suggested that artemisinin, in addition to killing parasites, has a direct action on differentiated erythrocytes other than definitive hematopoietic stem cells and causes erythroid apoptosis by interfering with the heme biosynthesis pathway in zebrafish and human cells.

Zebrafish hematopoiesis occurs in two successive waves, primitive and definitive hematopoiesis, similar to hematopoiesis in other vertebrates. Primitive hematopoiesis starts ~12 hours postfertilization (hpf) [1–3]. Instead of the extraembryonic yolk-sac blood island in mammals, this process in zebrafish occurs in two intraembryonic regions, the rostral blood island and the intermediate cell mass. The rostral blood island is derived from the anterior lateral plate mesoderm, producing *spi1*$^+$ myeloid progenitors, whereas the intermediate cell mass is derived from the posterior lateral plate mesoderm and generates *gata1*$^+$ erythroid progenitors [4]. Later, some *lmo2*$^+$ multipotential erythromyeloid progenitors appear in the caudal hematopoietic tissue but are quickly replaced by hematopoietic stem cells (HSCs) differentiated from hemogenic endothelium in the ventral wall of dorsal aorta, which is just like the aorta gonad mesonephros in mammals, marking the onset of definitive hematopoiesis in zebrafish [4–6]. From 3 to 4 days post fertilization (dpf), lymphoblasts gradually settle in the thymus via the circulation and HSCs in the kidney, remaining the source of blood cells throughout the whole life of zebrafish. Although the hematopoietic sites differ spatially between mammals and zebrafish, the underlying transcriptional mechanisms are evolutionally conserved [3]. Primitive hematopoiesis is regulated by transcription factors such as stem cell leukemia (scl/tal1), gata1, pu.1, and lmo2, and the initiation of definitive hematopoiesis needs the transcription factors *runx1* and *cmyb* [7].

Polycythemia vera (PV) is a myeloproliferative blood cancer in which the bone marrow makes too many red blood cells (RBCs) [8]. In 2005, Vainchenker and colleagues discovered the Janus kinase 2 (JAK2) mutation JAK2V617F in patients that causes the typical myeloproliferation characteristic of PV, thrombocythemia and myelofibrosis. The enhanced activity of JAK2V617F makes HSCs

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sensitive to hematopoietic growth factors, resulting in myeloproliferation [9]. In zebrafish, the counterpart of human JAK2V617F is jak2aV581F, which enhances the production of RBCs, making it a good zebrafish model of PV [10].

Artemisinin, also known as Qinghaosu, is widely used in antimalarial therapy. Artemisinin-basd combination therapy is recommended as the primary treatment for all cases of Plasmodium falciparum malaria [11]. Compared with other antimalarial drugs like chloroquine, artemisinin derivatives have been proved to be remarkably efficient and safe in clinical trials [12]. Animal trials have shown that one artemisinin derivative, dihydroartemisinin, affects erythrocytes at lower doses during yolk-sac hematopoiesis [13]. In recent years, cases of severe hemolytic anemia have been reported after parenteral artemisinin treatment, but the underlying cellular and molecular mechanisms are still unclear [14].

The zebrafish is an ideal model organism for small-molecule screening; its small size, fecundity, and rapid embryonic development make it suitable for high-throughput in vivo screening in 48- or 96-well plates [15,16]. External fertilization and development make zebrafish embryos accessible to small-molecule treatment and anesthetics [17]. Most importantly, since the level of conservation is rather high between mammals and zebrafish, studies on zebrafish have good potential for translation to mammalian systems. Here, by performing a small-molecule screen of a library containing 923 preclinical or intermediate drugs, we demonstrated that artemisinin suppressed erythrocytes in zebrafish embryos and differentiated human K562 erythrocytes through the activation of both mitochondria- and cell death receptor–mediated apoptosis.

Materials and methods

Zebrafish maintenance

Zebrafish were maintained and bred as described in the Zebrafish Book [18]. Tg(gata1:DsRed), Tg(CD41:eGFP), and Tg(*flk1:*eGFP) fish used in this study were provided by Dr. Feng Liu (Institute of Zoology, Chinese Academy of Sciences, Beijing, China). The wild-type tupfel long fin (TL) and transgenic zebrafish were raised and handled in accordance with the guidelines of the Peking University Animal Care and Use Committee, fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

O-dianisidine staining

We applied 500 μL fresh staining solution (2 mL of 14% o-dianisidine solution; 500 μL of 0.1 mol/L NaOAc, pH 4.5; 2 mL of deionized H_2O; 100 μL of H_2O_2) to live, dechorionated embryos in glass vials after removing as much of the E3 medium as possible. Embryos were incubated with staining solution in the dark for 20 min and then washed three times with deionized H_2O. Stained embryos were fixed in 4% paraformaldehyde for at least 1 hour at room temperature, and then stored in phosphate buffered saline Tween-20 (PBST) at 4°C or in MeOH at −20°C.

In vitro synthesis of antisense RNA probes

Digoxigenin-labeled antisense RNA probes were synthesized by in vitro transcription according to a standard protocol. Whole-mount in situ hybridization was performed as described in the Zebrafish Book [18].

Apoptotic assay by terminal deoxynucleotidyl transferase dUTP nick end labeling

Paraformaldehyde-fixed, dechorionated embryos were permeabilized with 10 μmol/L protease K (Roche, Indianapolis, IN), and then stained with the In Situ Cell Death Detection Kit, TMR red (Roche) or In Situ Cell Death Detection Kit, Fluorescein (Roche). Tg(gata1:DsRed) and Tg(*flk1:*eGFP) transgenic embryos were used to label red blood cells and vascular endothelial cells, respectively.

Cell culture

K562 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing 10% fetal bovine serum, under humidified conditions and 5% CO_2 at 37°C. Differentiation of blood cells was induced with 50 μmol/L hemin (Sigma-Aldrich, St. Louis, MO); half were treated with 50 μmol/L artemisinin and half with dimethyl sulfoxide (DMSO) as controls.

Caspase activity assays

K562 cells were collected after 36 hours of artemisinin or DMSO treatment in the presence or absence of 50 μmol/L hemin. After washing three times with phosphate-buffered saline (PBS), cells were suspended in PBS and counted. The activities of caspase 8 and caspase 9 were determined by Caspase-Glo 8 and 9 assays (Promega) according to the manufacturer's instructions.

Western blot

For Western blot analysis, protein extracts were electrophoresed on 12% NuPAGE gel and transferred to polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline/0.1%Tween-20 and incubated overnight with antiactive caspase 8 (Cell Signaling Technology) and anti-cleaved caspase 8 or 9 (Cell Signaling Technology). Membranes were washed and then incubated for 2 hours with secondary antibody in Tris-buffered saline/0.1%Tween-20 containing 5% nonfat dry milk. Detection was carried out using an enhanced chemiluminescence (ECL) Western blotting substrate Promega (Madison, WI) and quantified by scanning laser densitometry using ImageJ.

Library preparation for strand-specific poly(A)-positive RNA-sequencing

Wild-type control embryos, and embryos treated with artemisinin from 6 to 24 hpf, or embryos treated with artemisinin from 24 to 36 hpf, were collected and rinsed with precooled PBS. Total RNA was extracted from ∼25 embryos at 36 hpf using an RNeasy Microarray Tissue Mini Kit Qiagen (Hilden, Germany). Poly(A)-positive RNA was purified from 5 μg of total RNA (RNA integrity number ≥ 7.5) with a Dynabead mRNA purification kit Invitrogen (Waltham, Massachusetts), following the manufacturer's instructions. A strand-specific RNA-sequencing library was then prepared using the deoxy-UTP approach as reported previously [19]. Amplified materials were loaded onto a flow-cell,