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Contrasting intra- and extracellular distribution of catalytic ferrous iron in ovalbumin-induced peritonitis

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ABSTRACT

Iron is an essential nutrient for every type of life on earth. However, excess iron is cytotoxic and can lead to an increased cancer risk in humans. Catalytic ferrous iron [Fe(II)] is an initiator of the Fenton reaction, which causes oxidative stress by generating hydroxyl radicals. Recently, it became possible to localize catalytic Fe(II) *in situ* with a turn-on fluorescent probe, RhoNox-1. Here, we screened each organ/cell of rats to globally evaluate the distribution of catalytic Fe(II) and found that eosinophils showed the highest abundance. In various cells, lysosomes were the major organelle, sharing ~40–80% of RhoNox-1 fluorescence. We then used an ovalbumin-induced allergic peritonitis model to study the dynamics of catalytic Fe(II). Peritoneal lavage revealed that the total iron contents per cell were significantly decreased, whereas an increase in the number of inflammatory cells (macrophages, neutrophils, eosinophils and lymphocytes) resulted in an increased total iron content of the peritoneal inflammatory cells. Notably, macrophages, eosinophils and neutrophils exhibited significantly increased catalytic Fe(II) with increased *DMT1* expression and decreased *ferritin* expression, though catalytic Fe(II) was significantly decreased in the peritoneal lavage fluid. In conclusion, catalytic Fe(II) *in situ* more directly reflects cellular activity and the accompanying pathology than total iron does.

1. Introduction

No living thing on earth can survive without iron, but iron is a double-edged sword. Whereas deficiency causes anemia, the excess of iron is a risk for cancer and other various diseases in higher animals [1–3]. Over the past two decades, studies have revealed various novel molecules that are associated with iron absorption and transfer as well as their regulation at the transcriptional, post-transcriptional and protein degradation levels [4,5].

Because there is no active pathway to excrete iron from higher animals, local or global excess iron is observed in various different pathologies, including bacterial/viral infection, foreign body exposure [6], autoimmunity-associated inflammation and repeated hemorrhage in closed space [7]. Though distinct molecular mechanisms are involved in each case, catalytic (labile) ferrous iron, i.e., Fe(II), has been considered an initiator of the Fenton reaction, which generates the most reactive chemical species, hydroxyl radicals, *in vivo* [8]. Although the detection of oxidatively modified products became available during the 1980s and 1990s [9], the detection of catalytic Fe(II) *in situ* was not previously available.

In 2013, Hirayama et al. developed a fluorescent probe, RhoNox-1, to specifically detect catalytic Fe(II) [10], and we showed its applicability to frozen sections [11] based on a ferric nitrilotriacetate-induced renal carcinogenesis model [12–14]. Here, we comprehensively stained tissues in rats with RhoNox-1, which revealed that eosinophils have the highest level of catalytic Fe(II). Further experiments using ovalbumin (OVA) in a rat peritonitis model revealed a distinct altered distribution of catalytic Fe(II) in inflammatory cells.

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2. Materials and methods

2.1. Reagents

RhoNox-1 was synthesized as described [10], preserved at −80 °C until use, and dissolved in dimethyl sulfoxide to produce a 10 mM solution. It was further diluted with 10 mM phosphate-buffered saline (pH 7.2; PBS) and was used within a single day.

2.2. Animal experiments

The animal experiment committee of Nagoya University Graduate School of Medicine approved the following experiments. Fischer-344 rats were obtained from SLC Japan (Shizuoka, Japan). The OVA-induced allergic peritonitis model was produced as previously described [15–17]. Briefly, male Fischer-344 rats (6 weeks; 140–150 g body weight) were injected with an intraperitoneal injection of a mixture of 1 mg OVA (Thermo Fisher Scientific, Waltham, MA, USA) and 25 mg Alum (Sigma-Aldrich, St. Louis, MO, USA) three times for 3 consecutive days. Three weeks after sensitization, the rats were injected intraperitoneally with 1 mg OVA only (Fig. 1A). We collected peripheral blood (PB) and peritoneal lavage [PL; 30 ml physiological saline, containing 200 μM tris (2-pyridylmethyl) amine (Sigma Aldrich)] 48 h after the final exposure as previously described [18]. PB was analyzed with VetScan HM5 (Abaxis; Union City, CA, USA). PL samples underwent centrifugation (720 × g, 5 min) to collect cells, to which RhoNox-1 was added to a final concentration of 10 μM; the samples were then incubated for 1 h at 37 °C and analyzed with PowerScan4 (DS Pharma Biomedical, Osaka, Japan).

2.3. Histological detection of catalytic Fe(II)

Untreated control or peritonitis model rats were euthanized at 7–9 weeks of age. Each organ was dissected and embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA) with dry ice-cooled acetone as previously described [11]. Briefly, frozen sections of 8-μm thickness were prepared with a cryostat on a glass slide, fixed in 20% formalin in methanol for 3 min and washed with PBS five times. Then, 200 μl of 10 μM RhoNox-1 was placed on the specimens and incubated for 30 min at 37 °C in a dark chamber, followed by counterstaining with Hoechst33342 (Thermo Fisher) and observation with a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). It was possible to preserve the frozen sections prior to RhoNox-1 reaction at 4 °C for at least 24 h after cutting. For the quantitation of catalytic Fe(II), each image was divided into RGB elements, to which the ImageJ version 1.47 software (http://www.rsb.info.nih.gov/ij/) was applied: the blue component was used for the nucleus, and the red component was used to examine catalytic Fe(II). Integration of the red color (RhoNox-1) divided by the number of nuclei in the analyzed area with a 100× oil-immersion objective lens was calculated as arbitrary units (AU). At least three random areas were selected for each analysis.

2.4. Flow cytometry and cell sorting

Collected cells from Section 2.2 were rinsed by RPMI1640 without phenol red (Wako, Osaka, Japan), centrifuged (720 × g, 5 min) and suspended in 1-ml RPMI1640 without phenol red. The cells were counted with a hemocytometer. RhoNox-1 solution (final concentration 10 μM) was added to a tube containing cells and incubated in the dark at 37 °C for 30 min, after which Hoechst33342 (final concentration 100 μM) was added. After 30 min, the cells were centrifuged (180 × g, 5 min) and suspended in Hank's Balanced Salt Solution (HBSS) without phenol red (Wako). Cell analysis and sorting were carried out using FACS Aria II (BD Biosciences; San Jose, CA, USA). At first, Hoechst33342-negative cells were removed as dead cells. We observed 4 subsets of living cells with forward scatter (FSC) and side scatter (SSC), each of which was sorted (post-sorting purity > 90%) and underwent iron measurement analysis. Hoechst33342 and RhoNox-1 were excited by 375 nm and 488 nm, respectively. Fluorescent signals were measured at 450 nm (450/50) for Hoechst33342 and at 575 nm (575/30) for RhoNox-1. All flow cytometric data were analyzed using FACSDiva version 8.0.1.

2.5. Cell culture experiments

The HL-60 leukemia cell line and IMR-90-SV human fibroblast cell line, derived from embryonic tissue with SV40 immortalization, were from RIKEN CELL BANK (Ibaragi, Japan). The Met5A human mesothelial cell line, with SV40 transformation, was from ATCC (Manassas, VA, USA), and the NHLF human fibroblast cell line was from Lonza (Walkersville, MD, USA). RPMI1640, containing 10% fetal bovine serum (Biowest, #S1820; Nuaillé, France) and 1% antibiotic-antimyocotic (Gibco, #15240-062; Grand Island, NY, USA), was used in a humidified incubator at 37 °C with 5% CO_2. The cells were used within two months after thawing. Phorbol 12-myristate 13-ascetate (PMA; Wako) was diluted to 160 μM with acetone and preserved at −20 °C. HL-60 cells were incubated with 16 nM PMA for 48 h for differentiation into macrophages [19].

2.6. Western blotting

The cells were lysed with a lysis buffer (1 M NaCl, 50 mM Tris-HCl, 0.1% SDS, pH 8.0, 0.5% sodium deoxycholate and 1% NP-40) supplemented with a protease inhibitor cocktail (Roche; South San Francisco, CA, USA). Protein extraction, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses were performed as previously described [20], using anti-DMT-1 rabbit polyclonal antibody (Santa Cruz Biotechnology, sc-30120; Dallas, TX, USA) at a 1:1000 dilution; anti-TfR mouse monoclonal antibody (Thermo Fisher Scientific, 13–6800) at 1:1000; anti-SLC40A1 (ferroportin) rabbit polyclonal antibody (Abcam, ab58695; Cambridge, UK) at 1:500; anti-ferritin light chain rabbit polyclonal antibody (Abcam, ab69089) at 1:1000; anti-ferritin heavy chain rabbit polyclonal antibody (Santa Cruz Biotechnology, sc-25617) at 1:200; and anti-β-actin mouse monoclonal antibody (Sigma-Aldrich) at 1:2000.

2.7. Intracellular localization of catalytic Fe(II)

For fluorescent confocal microscopy, cells were plated on 35-mm glass-bottom dishes, maintained in phenol red-free medium without FBS and imaged through a 100× oil immersion objective lens on a LSM 880 microscope (Carl Zeiss, Oberkochen, Germany). HL-60, NHLF, IMR-90-SV and Met5A were incubated with 10 μM RhoNox-1 and LysoTracker® Green DND-26 (200 nM) (Thermo Fisher Scientific) for 1 h at 37 °C. After incubation, the cells were washed with RPMI1640 without phenol red and incubated with 25 nM MitoTracker® Deep Red FM (Thermo Fisher Scientific) and 100 nM ERTracker™ Blue-White DPX (Thermo Fisher Scientific). The medium was changed to FluoroBrite™ DMEM (Thermo Fisher Scientific), and a Zeiss 880 equipped with a laser diode, an argon laser, a DPSS Laser and a HeNe Laser was used. ERTracker was excited with 405 nm, LysoTracker with 488 nm, RhoNox-1 with 561 nm and MitoTracker with 640 nm, using the standard beam splitter setting.

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