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Haemin-induced cell death in human monocytic cells is consistent with ferroptosis

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ABSTRACT

Background: Iron overload is a major issue for transfusion-dependent patients. Repeated transfusions result in the loading of large amounts of haem-derived iron on macrophages, and the haemin in turn induces cell death and the generation of reactive oxygen species (ROS) in both murine macrophages and human monocytic THP-1 cells. This haemin-induced cell death process has been shown to be iron-dependent. Thus, we hypothesized that haemin-induced THP-1 cell death is a result of ferroptosis, an iron-dependent mechanism of cell death regulation.

Material and methods: Human monocytic THP-1 cells were treated with haemin, and haemin-induced cell death and ROS generation were assessed using flow cytometry.

Results: Haemin-induced THP-1 cell death showed a necrosis pattern, and treatment with iron chelators suppressed both haemin-induced cell death and ROS generation. Treatment with ferrostatin-1, a ferroptosis inhibitor, suppressed haemin-induced cell death without affecting ROS generation, whereas erastin, a ferroptosis inducer, enhanced both haemin-induced cell death and ROS generation.

Discussion: Our findings support haemin-induced cell death as an example of ferroptosis. Therefore, ferroptosis inhibitors may be useful for the treatment or prevention of transfusion iron overload.

1. Introduction

Several diseases render patients to become dependent on regular transfusions, including congenital syndromes, such as thalassemia and sickle cell diseases, and acquired syndromes, such as aplastic anaemia and myelodysplastic syndromes (MDS). Iron overload is a major problem that negatively affects clinical outcome for these transfusion-dependent patients [1–4]. Iron induces cytotoxicity through the production of reactive oxygen species (ROS) [2–4]. Iron accumulation in parenchymal cells, such as hepatocytes, pancreatic beta cells, and cardiac myocytes, causes liver dysfunction, diabetes mellitus, and cardiac failure as the most serious adverse event in such patients [1–4].

The two major sites of iron accumulation are hepatocytes and reticuloendothelial cells (macrophages in the bone marrow and spleen, Kupffer cells in the liver). In hereditary haemochromatosis (HH), a representative disease that causes iron overload, hepatocytes are the first site of iron accumulation [1,2,5]. Deficiency in hepcidin, an iron-regulating hormone, causes uncontrolled iron absorption in enterocytes and iron efflux from macrophages, resulting in elevated plasma iron levels [1,2,5]. Once the transferrin in plasma is saturated with iron, non-transferrin-bound iron (NTBI) is produced, which is taken up by parenchymal cells, and the accumulated iron causes cytotoxicity; however, iron accumulation in macrophages does not occur in HH because of increased iron efflux [1,2,5].

In contrast, reticuloendothelial cells are the first site of iron accumulation in the case of transfusion-related iron overload. Transfusions load large amounts of iron on macrophages as haem-iron [1,3,4]. Macrophages phagocytose senescent and damaged red blood cells (RBCs) and take up haemoglobin released from RBCs and haem released from extracellular haemoglobin [6]. Haem is catabolized by haem oxygenase-1 (HO-1) into iron, carbon monoxide, and biliverdin, and the processed iron is stored in cytoplasmic ferritin [1,2]. Selective accumulation of iron in reticuloendothelial cells is relatively safe and protects parenchymal cells from iron overload [1]. When the amount of loaded iron exceeds the macrophage's protective capacity, iron accumulates in parenchymal cells and causes organ dysfunction similar to

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HH [1,2]. Therefore, macrophages play a major role in the pathophysiology of transfusion iron overload.

Despite recognition of the clinical relevance of haem-iron overload in macrophages during transfusion iron overload, there have been few detailed investigations of these specific effects and mechanisms conducted to date [7–12].

Bozza's research group has intensively studied the underlying factors driving haem-related cytotoxicity using murine peritoneal macrophages and revealed that haem induces non-apoptotic cell death and ROS production via two pathways: iron-mediated ROS production and toll-like receptor 4 (TLR4)-mediated tumour necrosis factor (TNF) production [8–10]. Moreover, Bozza's group further demonstrated the iron-dependence of this haem-induced macrophage cell death [8,9], which led us hypothesize that this effect is an example of ferroptosis, an iron-dependent non-apoptotic cell death mechanism originally proposed by Stockwell et al. in 2012 [13]. A hallmark of ferroptosis is iron-dependent lipid peroxidation, which can be inhibited by iron chelators, and peroxidation inhibitors (i.e. ferroptosis inhibitors) such as ferrostatins and liproxstatins [13–16]. The haem-induced macrophage cell death described by Bozza's group for murine peritoneal macrophages appears to be largely consistent with the known process of ferroptosis, except for the dependence on TLR4 pathway. Indeed, we previously demonstrated that haemin induces non-apoptotic cell death and ROS generation in human monocytic THP-1 cells [17]. Haemin is the oxidized form of haem and the major component of extracellular haem *in vivo* [6].

In the present study, to test our hypothesis that haemin-induced cell death is an example of ferroptosis, we examined the effects of a ferroptosis inhibitor and inducer on haemin-induced THP-1 cell death.

2. Materials and methods

2.1. Cell culture

Human monocytic THP-1 cells, purchased from DS Pharma Biomedical (Osaka, Japan), were maintained in RPMI1640 medium supplemented with 10% foetal bovine serum (FBS; Hyclone, South Logan, Utah, USA) in a 5% CO_2 atmosphere at 37 °C.

2.2. Treatment with haemin and other reagents

THP-1 cells were washed with phosphate-buffered saline (PBS) twice to remove the FBS. The cells were then suspended in RPMI1640 medium at a density of 1×10^6 cells/ml and 1 ml of the cell suspension was seeded in each well of a 24-well plate. Haemin (Sigma, St. Louis, MO, USA), stocked in dimethyl sulphoxide (DMSO) at 5 mmol/l, was added to the wells and incubated in 5% CO_2 at 37 °C.

To examine the relationship between ROS generation and cell death induction, THP-1 cells were pretreated with either *N*-acetyl cysteine (NAC; Sigma), an inhibitor of ROS-mediated oxidation, or diphenyleneiodonium chloride (DPI; Cayman Chemical, Ann Arbor, MI, USA), an inhibitor of NADPH oxidase, a key enzyme involved in ROS generation. NAC and DPI were added to the medium 1 h prior to haemin treatment.

To examine the roles of iron in haemin-induced cell death and ROS generation, THP-1 cells were pretreated with the iron chelators deferoxamine (DFO) or deferasirox (DSX), gifted by Novartis (Basel, Switzerland), prior to haemin treatment. To specifically examine the effects of haemin on cell death and ROS generation independent of iron, the cells were treated with protoporphyrin IX (PpIX; Sigma), which is a component of haemin but does not contain iron. THP-1 cells were treated with 0–80 μmol/l PpIX for 2 h. According to Figueiredo's method [9], PpIX was dissolved in 0.1 N NaOH, diluted with RPMI, and filtered immediately before use. The entire procedure was conducted in the dark to avoid light-induced free radical generation.

To examine whether haemin-induced THP-1 cell death is dependent on TLR4 and/or the RIP1/RIP3 pathway, the cells were pretreated with 10 μmol/l TAK-242 (Chemscene, Monmouth Junction, NJ, USA), a TLR4 signal inhibitor, or 20 μmol/l necrostatin-1 (Sigma), a RIP1 inhibitor. Moreover, we evaluated whether caspase-dependent apoptosis plays a role in the cell death mechanism by treating the cells with 10 μmol/l zVAD-fmk (MBL, Woburn, MA, USA), a pan-caspase inhibitor.

To examine the specific role of ferroptosis in haemin-induced cell death and ROS generation, the cells were treated with ferrostatin-1, a potent ferroptosis inhibitor that inhibits the lipid peroxidation reaction, and erastin, which is a ferroptosis-inducing agent [13–16]. THP-1 cells were pretreated with 0–10 μmol/l erastin (BioVision, Milpitas, CA, USA) or 0–40 μmol/l ferrostatin-1 (Sigma) for 1 h, followed by haemin treatment as described above for 2 h.

Fortes et al. [8,9] reported that 10% FBS suppressed haemin-induced cell death and ROS production, and serum contains the haemin-specific binding protein haemopexin as well as albumin, a major component of the serum protein content that can also bind haemin. Therefore, we examined the potential suppressive effects of albumin on haemin-induced cell death and ROS generation. The concentration of albumin in FBS was determined to be 2.3 g/dl with a clinical chemistry analyser (CA-90; FURUNO, Nishinomiya, Hyogo, Japan). Thus, the concentration of albumin in the 5% FBS used in this study was 0.1 g/dl, which was applied to the THP-1 cells prior to haemin treatment.

2.3. Human peripheral monocyte isolation and treatment with haemin

ACD-anticoagulated peripheral blood was collected from healthy volunteers after obtaining informed consent. The experiment was approved by the Kobe Tokiwa University research ethics committee.

Peripheral blood mononuclear cells (MNCs) were isolated from three healthy volunteers by density gradient separation using Ficoll Paque Premium (GE Healthcare Bio-Science AB, Uppsala, Sweden), according to the manufacturer's protocol. After washing the cells twice with PBS, the MNCs were suspended in RPMI1640 containing 10% FBS and incubated in 5% CO_2 at 37 °C for 2 h. Once the monocytes adhered to the plate, the medium and floating cells were removed, and the cells were washed four times with PBS. The monocyte layer was incubated with serum-free RPMI1640 and treated with haemin.

2.4. Measurement of haemin-induced cell death by Annexin V-FITC and propidium iodide (PI) staining

Cell death was detected by Annexin V-FITC and PI double staining (Annexin V-FITC Kit; Beckman Coulter, Marseille, France), according to the manufacturer's protocol, and analysed using flow cytometry (FCM; FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA). Annexin V-FITC (PromoKine, Heidelberg, Germany) was also used for some experiments. Annexin-V binds to phosphatidylserine, which is a marker of apoptosis, on the cell surface. Since viable cells are impermeable to PI, PI positivity is a marker of necrosis. After haemin treatment, the cells were washed with PBS and suspended in 100 μl of binding buffer. Annexin V-FITC and PI were added to the cells, which were further incubated on ice in the dark for 10 min. After the addition of binding buffer, the cells were analysed with FCM.

2.5. Measurement of haemin-induced ROS generation

Intracellular ROS generation was measured based on the oxidation of CM-H2DCFDA (Molecular Probes, Eugene, OR, USA), a ROS detection reagent, according to the manufacturer's protocol, and analysed using FCM. After haemin treatment, cells were washed with PBS, resuspended in RPMI1640 medium, and reseeded into 24-well plates. CM-H2DCFDA was added at a final concentration of 0.5 μmol/l and incubated in 5% CO_2 at 37 °C for 30 min. Cells were washed with PBS, and ROS generation was analysed using FCM.