主論文の要旨

Embryonal erythropoiesis and aging exploit ferroptosis

〔 フェロトーシスは胎児造血と老化で利用される 〕

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【Introduction】

Ferroptosis is a type of regulated cell necrosis as a consequence of iron-dependent lipid peroxidation, which has led to the revised understanding of pathologies. However, up until now a physiological meaning of ferroptosis has not been established likely due to the lack of easy-to-perform and reliable methodologies to directly monitor ferroptosis in physiological and pathophysiological contexts. Here, we set out to search for a proper strategy to discover "physiological ferroptosis". We previously found that 4-hydroxy-2-nonenal (HNE) is the most sensitive marker among lipid peroxidation products and developed five different monoclonal antibodies against hemiacetal structure of Michael adducts in HNE-modified proteins. We started from reevaluating those antibodies and found that HNEJ-1 presents the most promising candidate for monitoring ferroptotic events in tissues.

【Methods】

Evaluation of antibodies for ferroptosis detection

1) Ferroptosis: HT1080 fibrosarcoma cells were treated with erastin in the presence or absence of ferrostatin-1(Fer-1) or deferoxamine mesylate (DFO). Apoptosis: HT1080 cells were treated with staurosporine (STS). Oxidative stress-induced necrosis: HT1080 cells were treated with H_2O_2 in the presence or absence of Fer-1 or DFO. Immunohistochemistry detection of HNEJ-1~5, anti-cleaved caspase-3, anti-ACSL4 or anti-PTGS2 were applied to cells as indicated.

2) Immunofluorescence detection of HNEJ-1, anti-ACSL4 or anti-cleaved caspase-3 were assessed in HT1080 cells treated with erastin or RSL3 in the presence or absence of Fer-1 or DFO; or those treated with STS.

3) Intracellular localization of HNE was assessed by co-staining of MitoTracker and HNEJ-1 or incubation with HNEJ-1 without permeabilization in HT1080 cells treated with erastin.

4) Kidneys sections from Gpx4 cKO mice were kindly provided by Pro. Marcus Conrad, and kidneys from wild-type C57BL/6 mice served as a control. For the study of acute renal damage, kidneys were harvested 3 hours after a single intraperitoneal injection of ferric nitrilotriacetate (Fe-NTA; 15 mg iron/kg) using 6-week-old male Wistar rats. Immunohistochemistry detection of HNEJ-1, anti-ACSL4 or anti-PTGS2 were assessed in kidney sections as indicated.

Study of physiological ferroptosis

1) For the study of physiological development, Fischer-344 rats of different ages (E9.5, E13.5, E15.5, E18.5, new born, weaning age, young adults, adults and the aged) were euthanized. Berlin blue staining or immunohistochemistry of HNEJ-1 antibody or anti-CD68 of the organs were exanimated as indicated.

2) 3-weeks-old male SAMP8 mice were randomly divided into three groups: CO group, fed a normal diet; HF group, fed a high-fat diet; and CHR group, fed a carbohydrate-restricted diet. Immunohistochemistry of HNEJ-1 or Anti-8-OHdG antibody were applied to the mouse skin harvested at 50 weeks of age.

3) Pregnant rats (day 13) were intraperitoneally injected with 10 mg/kg Lipro-1 consecutively for 4 days. Control groups were administrated with Ringer's lactate solution in the same manner. At day 18.5, embryos were harvested and fixed for HE staining or for blood collection. The expression of Lamin B and TfR1 were assessed by western blot.

【Results】

1) HNEJ-1 is useful for detecting ferroptosis by immunohistochemistry

Among the five antibodies, HNEJ-1 showed both high sensitivity and specificity in erastin-treated cells. Furthermore, HNEJ-1 exhibited specific immunostaining in ferroptosis models triggered by two distinct inducers, as confirmed by ACSL4 immunostaining (Figure 1). HNEJ-1 immunostaining did not increase during apoptosis in comparison to cleaved caspase-3 immunostaining in STS-induced apoptotic cells. We observed moderately increased positivity of HNEJ-1 in H_2O_2-induced necrotic cells with the increased expression of ACSL4 (Figure 2).

2) Subcellular localization of HNE-modified proteins recognized by HNEJ-1

When ferroptosis was induced, colocalization coefficients and weighted colocalization coefficients were increased both for HNEJ-1 and Mitotracker, indicating an increased immunostaining of HNEJ-1 in mitochondria. Moreover, we detected an increase in plasma membrane immunopositivity of ferroptotic cells (Figure 3).

3) Detection of ferroptosis with HNEJ-1 in animal models

Positivity of HNEJ-1 in Gpx4 cKO kidneys in comparison to that of wild-type confirmed the usefulness of HNEJ-1. Intense immunopositivity of HNEJ-1 in the renal proximal tubules in comparison to the control kidney was found in renal ferroptosis model by Fe-NTA. We observed increased expression of both ACSL4 and PTGS2 in these two renal ferroptosis models (Figure 4).

4) Ferroptosis increases with age in various organs

There was an age-dependent increase in ferroptotic cells in the kidney, spleen, liver, ovary and uterus, which was accompanied by substantial iron accumulation in the physiological processes. In the aged kidney, HNEJ-1 showed cytoplasmic immunostaining in the majority of proximal tubular cells. In the aged spleen, liver, ovary and uterus, HNEJ-1 showed immunostaining mainly in macrophages/Kupffer cells, as indicated by CD68

immunostaining, whereas there was also positivity in the stroma of ovary and in uterus myometrium as well as parenchymal cells (Figure 5&6).

5) Ferroptosis in skin senescence model

An increase in the indicators of skin senescence, atrophic epidermal cells with hyalinized collagen, was observed in HF group as compared to CTRL group, which was further increased in CHR group. We applied HNEJ-1 or 8-OHdG on the epidermis, and found that ferroptotic cells were significantly increased in the HF and CHR groups in comparison to the CTRL group (Figure 7).

6) Ferroptosis is involved in embryonic erythropoiesis

At the stage of E9.5, we observed ferroptosis in extra-embryonic endodermal component of visceral yolk sac and trophoblast giant cells. At E13.5, nucleated erythrocytes showed signs of ferroptosis. Of note, not only the level of HNE modification in erythrocytes but also the fraction of HNE-positive erythrocytes subsequently decreased as they enucleated during the process of maturation. Ferroptosis inhibitor Lipro-1 elevated the percentage of nucleated erythrocytes in comparison to those from control groups. Lamin B level was increased by immunoblot in the Lipro-1 group compared to the control groups, whereas no significance of TfR1 level was observed (Figure 8).

【Discussion】

In the present study, ferroptosis was observed in the extraembryonic endodermal component of visceral yolk sac, which was reportedly to influence the differentiation and development of blood islands and vessels. Moreover, ferroptotic cell death was observed in trophoblast giant cells (TGCs). Prolactin-like protein family members, exclusively expressed in TGCs in particular, were secreted for the regulation of hematopoiesis. Although erythrocytes are characterized by high iron requirements to sustain hemoglobin synthesis, the role of ferroptosis in erythropoiesis has not been clearly shown. In a study using mice with hematopoietic cell-specific GPX4-deficiency, ineffective erythropoiesis was observed and the maturation of reticulocytes to erythrocytes was defective. However, a recent study revealed the role of GPX4 in the human erythroblast enucleation in a ferroptosis-independent manner, which indicated its intricate role as a ferroptosis regulator in the process of erythropoiesis. Our results revealed a high level of HNE modification in nucleated erythrocytes. Notably, HNE modification in erythrocytes subsequently decreased according with enucleation during the maturation process. Ferroptosis inhibitors delayed embryonic erythropoiesis, suggesting a positive role of lipid peroxidation during the maturation of erythrocytes.

One major outstanding question is whether ferroptosis is adaptive or indicative of

biochemical failure in cells. Here, we observed an age-dependent increase in ferroptosis in the kidney, spleen, liver, ovary and uterus, which was accompanied by iron accumulation in Fischer-344 rats detected by HNEJ-1. Because the principal role of red pulp macrophages in spleen and Kupffer cells in liver is to actively phagocytose senescent erythrocytes, macrophage ferroptosis with significant iron accumulation, may regulate and can be a marker of aging. Furthermore, ferroptosis was significantly higher in the epidermis of SAMP8 mice, fed with high-fat or carbohydrate-restricted diets, suggesting that they are aggravating factors of skin aging. Sublethal concentration of HNE skin fibroblasts has an impact on the proliferative capacity of fibroblasts during in vitro aging. These results stress the involvement of ferroptosis in physiological senescence.

【Conclusion】

HNEJ-1 is useful for screening ferroptotic events in formalin-fixed paraffin-embedded specimens in addition to other reported ferroptosis markers, such as ACSL4 and PTGS2. Ferroptosis is involved in embryonic hematopoiesis/erythropoiesis and aging.