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Iron regulatory protein 2 in ovarian endometrial cysts

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ABSTRACT

Ovarian endometrial cysts cause some kinds of ovarian cancer, and iron is considered as one factor of carcinogenesis. In contrast, hypoxia is associated with progression, angiogenesis, metastasis, and resistance to therapy in cancer. We investigated hypoxia-induced perturbation of iron homeostasis in terms of labile iron, iron deposition, and iron regulatory protein (IRP) in ovarian endometrial cysts. Iron deposition, expression of IRPs, and a protein marker of hypoxia in human ovarian endometrial cysts were analyzed histologically. The concentration of free iron and the pO_2 level of the cyst fluid of human ovarian cysts ($n = 9$) were measured. The expression of IRP2 under hypoxia was investigated in vitro by using Ishikawa cells as a model of endometrial cells. Iron deposition and the expression of IRP2 and Carbonic anhydrase 9 (CA9) were strong in endometrial stromal cells in the human ovarian endometrial cysts. The average concentration of free iron in the cyst fluid was 8.1 ± 2.9 mg/L, and the pO_2 was 22.4 ± 5.2 mmHg. A cell-based study using Ishikawa cells revealed that IRP2 expression was decreased by an overload of Fe(II) under normoxia but remained unchanged under hypoxia even in the presence of excess Fe(II). An increase in the expression of IRP2 caused upregulation of intracellular iron as a result of the response to iron deficiency, whereas the protein was degraded under iron-rich conditions. We found that iron-rich regions existed in ovarian endometrial cysts concomitantly with the high level of IRP2 expression, which should generally be decomposed upon an overload of iron. We revealed that an insufficient level of oxygen in the cysts is the main factor for the unusual stabilization of IRP2 against iron-mediated degradation, which provides aberrant uptake of iron in ovarian endometrial stromal cells and can potentially lead to carcinogenesis.

1. Introduction

Iron, especially labile ferrous iron (Fe^{2+}), causes oxidative stress via the production of reactive oxygen species by Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO- + HO\cdot$) and is often involved in carcinogenesis [1]. Following the development of a highly specific turn-on fluorescent probe (RhoNox-1) specific to Fe^{2+}, several models of carcinogenesis were investigated regarding involvement of labile Fe^{2+} [2–6]. The presence of ovarian endometrial cysts may lead to certain kinds of ovarian cancer (e.g., clear cell carcinoma and endometrioid adenocarcinoma), and dysfunction of iron homeostasis has been considered as a factor of carcinogenesis [7–10].

Contrastingly, hypoxia is associated with progression, angiogenesis, metastasis, and resistance to therapy in cancer [11]. It was reported that hypoxia-inducible factor 1 (HIF-1) plays an important role in the carcinogenesis of ovarian endometrial cysts [12] and is correlated with tumor growth in ovarian cancer [13].

In the course of our research on the carcinogenesis of ovarian endometrial cysts induced by iron and hypoxia, we found that the synergistic roles of iron and hypoxia during carcinogenesis could be reasonably explained by considering the usual stabilization of iron regulatory protein (IRP) 2. Therefore, we investigated the hypoxia-induced perturbation of iron homeostasis in ovarian endometrial

Abbreviations: DFO, deferoxamine; FAS, ferrous ammonium sulfate; FBS, fetal bovine serum; Fe^{2+}, ferrous iron; HIF-1, hypoxia-inducible factor 1; IRP, iron regulatory protein; CA9, Carbonic anhydrase 9; PBS, phosphate buffered saline; Tfr, transferrin receptor.

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cysts.

2. Materials and methods

2.1. Human samples

The ethics committee of the Gifu University Graduate School of Medicine approved the experiments. Written informed consent was obtained from all patients. The human ovarian endometrial cysts were obtained from patients undergoing laparoscopic cystectomy, and normal endometrial tissue was obtained from the uterus of patients undergoing hysterectomy for CIN3. The fluid from the ovarian cysts (n = 9) was collected during laparoscopic surgery using a SAND balloon (Hakko, Kagawa, Japan) before cyst rupture.

2.2. Histological analysis of catalytic Fe^{2+}

Frozen sections were cut at a thickness of 8 μm with a cryostat onto MAS-GP type A coated slide glass (Matsunami, Osaka, Japan), air dried for 5 min, fixed for 3 min in 20% formalin in methanol buffered with 10 mM phosphate-buffered saline (PBS), and washed for 5 min in PBS. RhoNox-1 was prepared according to the reported procedure [3]. RhoNox-1 was stored at −80 °C and dissolved in dimethylsulfoxide to produce a 10 mM solution, which was further diluted (1:1000) with 10 mM PBS (pH 7.2) before use to a final concentration of 10 μM. Thereafter, an ample amount of 10 mM RhoNox-1 was applied to the specimens, followed by incubation for 30 min at 37 °C in a dark box and a rinse in PBS. Then, the specimens were counterstained with Hoechst 33342, and images were captured with a BZ-9000 microscope (Keyence).

2.3. Perl's blue stain

Samples were fixed in 10% formalin and embedded in paraffin. Sections were cut at a thickness of 3 μm with a TU-213 microtome (Yamato, Saitama, Japan) onto MAS-GP type A coated slide glass. After deparaffinization, sections were flooded with equal parts of a mixture of ferrocyanide and hydrochloric acid for 1 h. After washing, the sections were counterstained with filtered neutral red stain for 5 min and dehydrated.

2.4. Immunohistochemistry

Samples were fixed in 10% formalin and embedded in paraffin. The sections were cut at a thickness of 3 μm with a TU-213 microtome onto MAS-GP type A coated slide glass. After deparaffinization, the sections were heated at 121 °C in Target Retrieval Solution (Dako, Tokyo, Japan) using an autoclave (ACV-3167N; Iwaki, Fukushima, Japan) for 1 min. Following blocking with Peroxidase-Blocking Solution (Dako, Tokyo, Japan), sections were incubated overnight at 4 °C with the following primary antibodies: Carbonic anhydrase 9 (CA9) (NB100-417, 1:1000; Novus Biologicals, CO, USA) and IRP2 (7H6, 1:100; Santa Cruz Biotechnology, CA, USA). After incubation with secondary antibody (LSAB2 kit; Dako, Fukushima, Japan), immune complexes were visualized using a DAB kit (Dako, Fukushima, Japan).

2.5. Ovarian endometrial cyst fluid

After collection of cyst fluid by using a SAND balloon and blood gas sampling kits (Terumo, Tokyo, Japan) during laparoscopic surgery, the fluid pO_2 level was measured immediately using an ABL 700 series system (Radiometer, Copenhagen, Denmark).

After the addition of 1 mL heparin, the samples were aliquoted and frozen at −80 °C. After dissolving the sample, the solution was centrifuged at 10,000 rpm for 10 min. The collected supernatants were used as the samples for assay. The concentration of free iron was measured by an iron assay kit (FE31M; Metallogenics, Chiba, Japan) according to the manufacturer's protocol. The optical density of 750 nm was measured by using a micro-titer plate reader.

2.6. Cell culture

Ishikawa cells (SIGMA-ALDRICH, Tokyo, Japan) were cultured in Dulbecco' s modified medium (Wako, Saitama, Japan) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Wako) under 5% CO_2 at 37 °C.

2.7. Hypoxic culture

Ishikawa cells were placed in a humidified (37 °C) hypoxic glove box with a gas mixing system. Hypoxic conditions were set in an INVIVO2 200 Physiological Cell Culture Workstation (Ruskinn, FL, USA) maintained at 0.5–1% O_2 and 5% CO_2 with a balance of nitrogen by GAS MIXER Q (Ruskinn). Lysates of the hypoxia-treated cells were prepared inside the box.

2.8. Western blotting

Protein (100 μg) was analyzed by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Proteins were detected using primary antibodies, IRP2 (7H6, 1:100, Santa Cruz Biotechnology) and GAPDH (FL-335, 1:500, Santa Cruz Biotechnology). Followed by reaction with anti-mouse IgG (sc-2005, 1:2000, Santa Cruz Biotechnology) for IRP2 and anti-rabbit IgG (sc-2004, 1:2000, Santa Cruz Biotechnology) for GAPDH, ECL Plus blotting detection reagents (GE Healthcare, IL, USA) were used according to the manufacturer's instructions.

3. Results

3.1. Catalytic Fe^{2+} in human ovarian endometrial cysts

We performed histological detection of deposited iron species with Perl's Prussian blue staining and of Fe^{2+} with RhoNox-1 in the frozen sections of human ovarian endometrial cysts (Fig. 1A). We found that the localization patterns of the deposited iron and the catalytic iron overlapped well.

3.2. Iron deposition and hypoxic region in human ovarian endometrial cyst tissue

The distribution of deposited iron was associated with the expression of IRP2 and CA9 in endometrial stromal cells in the ovarian endometrial cysts, whereas they were not detected in normal endometrium of the uterus (Fig. 1B). There was no significant difference in other iron regulatory factors (e.g., divalent metal transporter, transferrin receptor (TfR), ferroportin, ferritin) and IRP1 between the ovarian endometrial cysts and the normal endometrium of the uterus (data not shown).

3.3. Iron and oxygen levels in ovarian endometrial cyst fluid

The average concentration of free iron in the fluid of the ovarian endometrial cysts was 8.1 ± 2.9 mg/L, and the pO_2 was 22.4 ± 5.2 mmHg (see Table 1).

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