主論文の要旨

Carbonic anhydrase 9 confers resistance to ferroptosis/apoptosis in malignant mesothelioma under hypoxia

（Carbonic anhydrase 9 は低酸素下の悪性中皮腫においてフェロトーシスとアポトーシスへの抵抗性を与える）

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【Introduction】

Hypoxia and acidity provide microenvironment for selection under evolutionary pressure and proliferation in cancer cells. Carbonic anhydrases (CAs) are a superfamily of metalloenzymes present in all life kingdoms, equilibrating the reactions among CO_2, bicarbonate and H^+. CA9, a membrane-associated α-CA, has been a drug target for various cancers. Whereas iron is essential not only for cancer cells but also for all the lives on earth, little is known on the association among hypoxia, iron metabolism, extracellular acidity and redox regulation. Malignant mesothelioma (MM), an aggressive tumor with poor prognosis, is an intriguing model in that asbestos-associated pathogenesis includes excess iron environment during carcinogenesis. Re-analysis of rat asbestos-induced MM model revealed an inverse association between high *CA9* expression and survival. Here we used human MMs to identify the molecular events surrounding CA9 from the viewpoint of iron metabolism.

【Materials and Methods】

Human mesothelioma cell lines, ACC-Meso-1, NCI-H2373, NCI-H2052 and immortalized human mesothelial cell line, MeT-5A were used. Cells were cultured in both of normoxic and hypoxic conditions containing 10% fetal bovine serum under 5% CO_2 atmosphere. To evaluate the role of CA9 in mesothelioma cells, CA9 inhibitors, S4 and U104 were dissolved in dimethyl sulfoxide (DMSO) and treated with ACC-Meso-1 cells under hypoxia. Cell viability was accessed either with MTT or WST-1 assay. Iron-metabolism was analyzed by western blotting analyses. Catalytic Fe(II) was detected with a fluorescent turn-on probe, SiRhoNox-1. Cellular ROS levels were evaluated by staining with CM-H2DCFDA and MitoSOX; lipid peroxidation was quantified by incubating with BODIPY C11. Mitochondrial morphology was analyzed by confocal or transmission electron microscopy. To examine the consequence of CA9 inhibition in MM cells, Annexin V-FITC/PI staining and TUNEL assay were performed to evaluate apoptosis; a ferroptosis inhibitor (Fer-1) and an iron chelator, deferoxamine mesylate (DFO) were used to access ferroptosis.

【Results】

1. *Hypoxia promotes CA9 overexpression in MM cells with increased intracellular catalytic Fe(II)*

CA9 protein levels were significantly ($P < 0.05$ or $P < 0.01$) increased in all the three MM cell lines in response to hypoxia and all MM cell lines exhibited higher CA9 protein levels compared with MeT-5A (Fig. 1A). Iron-metabolism was also changed which exhibited decreased protein levels of iron transporters, TFRC and FPN-1(SLC40A1) with increased iron storage proteins, FTL and FTH. Additionally, IRP1/2 proteins were

increased in response to hypoxia (Fig. 1B). A significantly ($P < 0.001$) elevated catalytic Fe(II) signals were detected in lysosomes ($P < 0.01$) and mitochondria ($P < 0.001$) in ACC-Meso-1 cells in response to hypoxia (Fig. 1C).

2. *CA9-specific inhibitors delay proliferation and block migration of MM cells under hypoxia*

CA9-specific inhibitors, S4 or U104 treatment significantly ($P < 0.001$; both S4 and U104) decreased cell viability of ACC-Meso-1 cells at 48 and 72 h post treatment whereas vehicle treatment (DMSO) showed no effect on proliferation (Fig. 2A). Both S4 and U104 revealed anti-proliferative effects ($P < 0.001$ and $P < 0.01$, respectively) on non-carcinomatous mesothelial cells (MeT-5A) though the effects were milder than in ACC-Meso-1 cells (Fig. 2B).

3. *CA9 inhibition under hypoxia results in iron overload in lysosomes and mitochondria, inducing oxidative stress and triggering lipid peroxidation in MM cells*

CA9 inhibition in ACC-Meso-1 cells showed a dose-dependent decrease in CA9 expression under hypoxia. The protein levels of TFRC and FPN-1 were increased whereas those of FTL and FTH were decreased, consistent with up-regulation of iron-regulatory proteins, IRP1 and IRP2 (Fig. 3A). Furthermore, the level of catalytic Fe(II) ($P < 0.01$ and $P < 0.05$, S4 and U104, respectively)(Fig. 3B) was increased in response to CA9 inhibition which was accompanied by oxidative stress and increased lipid peroxidation (Fig. 3C, 3D, 3E), a surrogate markers for ferroptosis. The increased Fe(II) was found in both of lysosomes and mitochondria ($P < 0.001$) in response to CA9 inhibition in ACC-Meso-1 cells under hypoxia (Fig. 3F).

4. *CA9 inhibition under hypoxia leads to mitochondrial fission contributing to autophagy in MM cells*

S4-treated ACC-Meso-1 cells under hypoxia exhibited shortened ($P < 0.001$) mitochondrial length compared with the control group (Fig. 4A). S4-treated ACC-Meso-1 cells also presented diverse abnormities in mitochondria and mitophagy was detected (Fig. 4B). A significant ($P < 0.001$) increase in merged staining area of Mito and LysoTracker was found in S4-treated ACC-Meso-1 cells (Fig. 4C). Further confirmation of mitophagy was achieved by immunofluorescence staining of LC3B and MitoTracker which displayed a significantly ($P < 0.001$) increased LC3B and MitoTracker merge staining (Fig. 4D). LAMP-1, another marker for autophagosome and lysosome, was also augmented in response to S4 or U104 treatment accompanied by increased LC3B-II (lipidated form) in ACC-Meso-1 cells under hypoxia for 48 h (Fig. 4E).

5. ***CA9 inhibition induces both apoptosis and ferroptosis***

CA9 inhibition caused significant cell death detected by SYTOX Green at 48 h in ACC-Meso-1 cells under hypoxia (Fig. 5A). Both S4 and U104 induced apoptosis detected by analyses of Annexin V-FITC, TUNEL assay (Fig. 5B, 5C). To elucidate the involvement of ferroptosis, a ferroptotic (Fer-1) inhibitor and an iron chelator (DFO) were co-treated with S4 or U104 in ACC-Meso-1 cells under hypoxia. Fer-1 (3 μM) was significantly effective against S4 and U104 treatment ($P < 0.05$ and $P < 0.01$, respectively). DFO at a low concentration (0.5 μM) significantly ($P < 0.01$) attenuated S4-induced cytotoxic effect (Fig. 5D).

【Conclusion】

CA9 plays a pivotal role in the metabolism of MM cells under hypoxia, providing numerous merits, such as proliferation and migration, where iron homeostasis is maintained for their optimal growth with minimal oxidative cytotoxicity to avoid ferroptosis and apoptosis. Therefore, CA9 is an attractive molecular target for chemotherapy of MM, considering the present dismal therapeutic status. Because CAs have been a drug target for various pathologies, including cancers, glaucoma, obesity and Alzheimer's disease, re-exploration of other pathologies from the viewpoint of catalytic Fe(II) might be fruitful.