Research Paper

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

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ABSTRACT

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\textsubscript{2}, bicarbonate and H\textsuperscript{+}. 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. CA9 expression was significantly higher in MM cells than in MeT-5A mesothelial cells, which was further amplified under hypoxia (1\% O\textsubscript{2}) with increased catalytic Fe(II). CA9 suppression by inhibitors (S4 and U104) decreased viability and migration of MM cells, accompanied by overexpression of TFRC, IREB1/2 and FPN1(SLC40A1) and mitochondrial O\textsubscript{2}\textsuperscript{−} and lipid peroxidation. The eventual cell death was significantly inhibited by deferoxamine, ferrostatin-1 and Z-VAD-FMK, suggesting a mixed cell death of ferroptosis and apoptosis. Therefore, CA9 plays a role in equilibrating among hypoxia, iron metabolism and redox regulation in MM cells.

1. Introduction

Cancer is a leading cause of human mortality across the globe [1]. Hypoxia and extracellular acidity provide microenvironment for selection under evolutionary pressure and persistent invasive proliferation in cancer cells [2]. Malignant mesothelioma (MM) is a rare but highly aggressive cancer, exhibiting short median survival (∼13 months) [3,4]. MM derives from mesothelial cells, wrapping internal organs and lining the somatic cavities to provide a protective surface and to facilitate intracoelomic movement. Recently, pathogenic mechanisms linking asbestos-exposure [5] and catalytic Fe(II)-induced oxidative stress in MM carcinogenesis have been well established [6,7]. Hemoglobin adsorption on asbestos surface is a key phenomenon causing local iron overload [8–10], eventually leading to oxidative DNA damage in mesothelial cells due to the affinity of asbestos to histones [9,11]. Thus, repeated phlebotomy [12] or local iron removal [13,14] is suggested as a preventive measure against MM after exposure to asbestos, when therapeutic regimens for MM are still dismal [15].

Hypoxia is a microenvironmental hallmark in solid tumors, which is regarded as pO\textsubscript{2} < 10 mmHg (equivalent to < 1.3% O\textsubscript{2} in vitro) in comparison to pO\textsubscript{2} of 40–50 mmHg in peritoneal cavity [16]. Mass hypoxic regions have been well recognized in human MM [17,18], which may be endowing MM with various aggressive behaviors, including invasion, high proliferation rate [19] and a resistance to apoptosis [20], based on aberrant activation of a heterodimeric transcription factor, hypoxia inducible factor-1 (HIF-1) [21–23]. Of note, numerous iron metabolism-associated genes [24], including iron transport (Ex. TFRC [also as TFR1] and SLC11A2, encoding transferrin...
receptor and divergent metal transporter-1, respectively) [25–27]; iron storage (FTH, encoding ferritin heavy chain) [28] are under control by hypoxia-responsive elements in the promoter region. Hypoxia may upregulate with a postranslational mechanism the levels of iron regulatory protein 2 (IRP2), which can regulate the stability of each mRNA differentially to increase for TFRC and SLC11A2 and simultaneously to decrease for FTH [29]. Furthermore, recent studies suggest that hypoxia promotes cancer cells to acquire aggressive phenotypes through re-programming iron metabolism for more iron acquisition and retention [30,31]. However, it is still unclear why MM cells under hypoxia continue to show iron addiction without toxicity [7].

Carbonic anhydrases (CAs; EC 4.2.1.1) are a superfamily (CA1-16) of metalloenzymes present in all life kingdoms, equilibrating the reactions among CO2, bicarbonate and H+ [32]. Carbonic anhydrase 9 (CA9), a membrane-associated α-CA present in fish, rodents and primates [33], has been a drug target for various cancers [34]. CA9 is an inductively expressed gene in response to hypoxia in cancers [35,36]. In addition to intracellular pH regulation, extracellular acidity by CA9 appears to provide multiple merits for cancer cells [2]. For example, acidic pH can facilitate extracellular matrix remodeling through over-expressing and activating reorganizational proteases, such as matrix metalloproteinases [37], and further may aid evasion of apoptosis by adopting the pro-survival Bcl-2 family expression pattern [38]. CA9 indeed contributes to the migration and invasion of tumor cells through enhancing MMP14-mediated collagen degradation in breast cancer cells [39]; CA9 overexpression can increase metastatic potential through Rho-GTPase-related epithelial-mesenchymal transition in a cervical cancer cell line [40]; CA9 overexpression is associated with a reduced Th1 response and worse overall survival in metastatic melanoma and basal-like breast cancer [41]. In the present study, we used human MM cells to identify the molecular events surrounding CA9 from the viewpoint of iron metabolism, considering a recent report that crocidolite or point of iron metabolism, considering a recent report that crocidolite or iron storage (FTH, encoding ferritin heavy chain) [28] are under control by hypoxia-responsive elements in the promoter region. Hypoxia may upregulate with a postranslational mechanism the levels of iron regulatory protein 2 (IRP2), which can regulate the stability of each mRNA differentially to increase for TFRC and SLC11A2 and simultaneously to decrease for FTH [29]. Furthermore, recent studies suggest that hypoxia promotes cancer cells to acquire aggressive phenotypes through re-programming iron metabolism for more iron acquisition and retention [30,31]. However, it is still unclear why MM cells under hypoxia continue to show iron addiction without toxicity [7].

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## 2. Materials and methods

### 2.1. Cell lines and culture conditions

Human mesothelioma cell lines, ACC-Meso-1 [43], NCI–H2373 and NCI–H2052, provided as gifts from Dr. Adi F. Gazdar, were used. Immortalized human mesothelial cell line, MeT-5A, was purchased from ATCC (Manassas, VA). Cells were cultured with RPMI-1640 (189–02025, Wako, Osaka, Japan) containing 10% fetal bovine serum (BioWest; Nuaillé, France) under 5% CO2 atmosphere. To generate hypoxic condition, cells were cultured in a Tri-Gas incubator (MCO-5M, PHCbi; Tokyo, Japan) achieved by nitrogen balanced atmosphere (94% N2, 1% O2, 5% CO2).

### 2.2. Chemicals

CA9 inhibitors, S4 (5577) and U104 (4540) were from Tocris Bioscience (Bristol, UK), which were dissolved in dimethyl sulfoxide (DMSO). Erastin (17754) was from Cayman Chemical (Ann Arbor, MI). Ferrostatin-1 (Fer-1; SML0583) was from Sigma-Aldrich (St. Louis, MO). Z-VAD-FMK (Z-VAD; S7023) was from Selleck. Deferoxamine mesylate (DFO; D9533) was from Sigma Aldrich (St. Louis, MO).

### 2.3. Antibodies

Mouse monoclonal anti-CA9 antibody (NBP1-51691) was from Novus Biologicals (Centennial, CO). Rabbit monoclonal anti-HIF-1α (ab51668), rabbit polyclonal anti-SLC40A1 (ab58695) and rabbit polyclonal anti-ferritin light chain antibody (ab69090) antibodies were from Abcam (Cambridge, UK). Rabbit polyclonal anti-ferritin heavy chain antibody (sc-25617) was from Santa Cruz Biotechnology (Dallas, TX). Mouse monoclonal anti-transferin receptor-1 antibody (13–6800) was from Thermo Fisher Scientific (Pittsburg, PA). Rabbit monoclonal anti-IRP1/IRP2 (20272S/37135), rabbit monoclonal anti-LAMP1 (9091), rabbit monoclonal anti-CA9 (NBP1-51691) and rabbit polyclonal anti-cleaved caspase-3 (9661) antibodies were from Cell Signaling (Danvers, MA). Mouse monoclonal anti-β-actin (A1978) was from Sigma Aldrich.

### 2.4. RNA isolation and quantitative RT-PCR

Total RNA was isolated from cultured cells by using RNeasy Plus Mini Kit (Qiagen; Hilden, Germany) and reversely transcribed into cDNA using SuperScript III First-Strand Synthesis kit (Invitrogen; Carlsbad, CA). Gene expression levels were quantified by Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) by using an Applied Biosystems Model 7300 Real-Time PCR System (Applied Biosystems; Waltham, MA). The mRNA levels were normalized by GAPDH (as internal reference) and calculated according to the 2−ΔΔCT method [44]. Multiple independent samples were used (N≥3), each of which was analyzed in duplicate. The primer sequences were as follows: human CA9, forward, 5′-GGGTGTCATCCTGACTGTTGTT-3′; reverse, 5′-CTTCT GTGCTGCCCTTCTCATC-3′ (product size, 309 bp); human DRP1, forward, 5′-CAAGGTGGCCGTAGGGATG-3′; reverse, 5′-CAGCAGTGA GGGAGATA-3′ (108 bp); human GAPDH, forward, 5′-CTGACTTCAA CGGAGACCC-3′; reverse, 5′-TGGCCAAATCTGTTGTACACC-3′ (109 bp).

### 2.5. Protein extraction and Western blot analysis

Protein lysate was prepared from cultured cells by using an ice-cold cell lysis buffer supplemented with a protease inhibitor cocktail (Roche;
2.0 × 10^3 cells were seeded per well in 96-well plates and incubated for 24 h. Cell viability assay

SLC40A1 (1:500), anti-LC3B (1:1000), anti-LAMP-1 (1:1000), anti-IRP1/IRP2 (1:2000), anti-ferritin light/heavy chain (1:1000), anti-β-actin (1:2000).

2.6. Cell viability assay

Cell viability was accessed either with MTT or WST-1 assay. 2.0 × 10^3 cells were seeded per well in 96-well plates and incubated for 16 h at 37 °C in normoxia (21% O2). After treatment with CA9 inhibitors under hypoxia for 24, 48 or 72 h, medium was replaced with fresh serum-free RPMI-1640 containing MTT or WST-1 reagent and incubated in normoxia for 2.5 h at 37 °C. Plates were scanned at 560 nm (MTT) or 450 nm (WST-1) with a Multiscan FC plate reader (Thermo Fisher Scientific).

2.7. Wound healing assay

The cells were seeded in 6-well plates and incubated in normoxia. After reaching confluence, wounds were inflicted by scratching with a sterile pipette tip for 200 μl across the monolayer, when the medium was replaced to remove cell debris. The cells were thereafter incubated under hypoxia with CA9 inhibitors for 24 h. The identical area of wound at the central area was measured at 0 and 24 h time points, respectively. The healing rate was determined by measuring wound ratio between the closure and the original areas, using Photoshop CS6 software (Adobe; San Jose, CA).

2.8. Cellular localization of catalytic Fe(II)

Catalytic Fe(II) was detected with a fluorescent turn-on probe, SiRhoNox-1 (FerroFarRed; Goryo Chemical, Sapporo, Japan), as previously described [46]. Cells (5 × 10^4) were cultured in normoxia on 35-mm glass bottom dish (Matsumani; Kishiwada, Japan) for 16 h at 37 °C. After treatment with CA9 inhibitors under hypoxia for 48 h, cells were co-stained with 5μM of SiRhoNox-1 and 200 nM of LysoTracker Green (Thermo Fisher Scientific) in FluoroBrite DMEM (A1896702, Gibco, Thermo Fisher Scientific) for 30 min at 37 °C in normoxia, followed by incubation with 75 nM of MitoTracker Red (Thermo Fisher Scientific) for another 30 min. We confirmed that 1-h incubation in FluoroBrite DMEM containing 0.1 mg/L (0.25 μM) Fe(NO3)3 scarcely affects iron metabolism of the cells within this limited period. Medium was replaced with new FluoroBrite DMEM for 30 min in normoxia at 37 °C. The cells were then collected and analyzed, using flow cytometer (Gallios BD, Beckman Coulter; Tokyo, Japan). Data was analyzed by Kaluza Analysis Software (Beckman Coulter).

2.10. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

After treatment with CA9 inhibitors under hypoxia for 48 h, cells were fixed with 4% paraformaldehyde for 10 min at RT and washed 3 times by PBS. TUNEL assay was performed, using apoptosis in situ detection kit (Wako) according to the manufacturer’s protocol, followed by detection with a light microscope.

2.11. Detection of ROS

After inhibition of CA9 in MM cells under hypoxia or 48 h, cellular ROS levels were evaluated by staining with 5μM of CM-H2DCFDA (Thermo Fisher Scientific, C6827) and with 5μM of MitoSOX (Thermo Fisher Scientific, M36008); lipid peroxidation was quantified by incubating with BODIPY C11 (Thermo Fisher Scientific, D3861) in FluoroBrite DMEM for 30 min in normoxia at 37 °C. The cells were then collected and analyzed, using flow cytometry (Gallios BD, Beckman Coulter) was used to analyze staining intensity.

2.13. Transmission electron microscopy

MM cells pre-treated with CA9 inhibitor (S4) was fixed with 2 mM glutaraldehyde in 1 mM PBS as previously described [47]. Transmission electron microscopy was performed with a JEM-1400PLUS (JEOL; Tokyo, Japan).

2.14. siRNA transfection

Cells (5 × 10^4) were seeded in 6-well plate and cultured in normoxia for 16 h. Two silencer select siRNAs to human CA9 (Invitrogen; s2270 and s224790) and one negative control siRNA (Invitrogen; 4390844) were used. siRNA complexes were generated by the use of RNAiMAX (Invitrogen; 137778) transfection reagent in Opti-MEM (Gibco; A1424801), followed by incubation at RT for 15 min. RNA and protein were extracted after siRNA treatment for 48 or 72 h under hypoxia.

2.15. Analysis of mitochondria morphology

Cells treated with CA9 inhibitor (S4) were stained with MitoTracker Red for 30 min in normoxia. Cells were fixed with 4% (w/v) paraformaldehyde for 10 min at RT, followed by incubation with ice-cold acetone for 5 min. Images were captured with a confocal microscope (LSM880, Carl Zeiss) and mitochondrial morphology was analyzed by Fiji distribution of ImageJ platform (MINA) according to the instructions [48].

2.16. Statistics

All statistical analyses were performed with GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Significance of difference was determined by unpaired t-test, one-way ANOVA or two-way
Fig. 1. CA9 overexpression in MM cells correlates with increased catalytic Fe(II) in response to hypoxia. (A) Differential overexpression of Cat(r)9 in asbestos-induced rat MM previously analyzed by expression array. Normal mesothelium (N = 2) / Met-5A cells (N = 2) in combination (N = 4) were used as a control; SM (N = 13) or EM (N = 9) represent sarcomatoid or epithelioid subtypes of malignant mesothelioma (MM) [6,49]. (B) Cat(r)9 overexpression in MM samples (shown in A plus biphasic subtype; N = 2; total N = 24) exhibited an inverse correlation with rat survival [6]. Pearson's correlation coefficient and corresponding P value is indicated. Quantitative RT-PCR (C) using GAPDH and western blotting (D) using β-actin, as corresponding internal standards, were performed to access CA9 mRNA and protein levels by using two human EM (ACC-Meso-1 and NCI-H2052) and one SM (NCI-H2373) cell lines in comparison to non-tumorous human mesothelial cell (MeT-5A) after 48 h-incubation in normoxia (N) or hypoxia (H; 1% O2). (E) Western blotting was performed to determine levels of iron metabolism-associated proteins in MM cells after 48 h-incubation in normoxia or hypoxia. (F) Differential levels of catalytic Fe(II) in normoxia or hypoxia of ACC-Meso-1 cells were determined by staining with SiRhoNox-1 (5 μM), followed by evaluation with a flow cytometer after 48 h-incubation. (G) Localization of catalytic Fe(II) in normoxic or hypoxic ACC-Meso-1 cells were analyzed by co-staining of SiRhoNox-1 (5 μM), LysoTracker (200 μM) and MitoTracker (75 μM) after 48 h-incubation and observation with a confocal microscope. Integrated intensity per cell (N = 7) was quantified by ZEN Imaging Software (ZEISS) (means ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001 vs each control unless indicated by bar). Refer to text for details. SM, sarcomatoid mesothelioma; EM, epithelioid mesothelioma; N, normoxia; H, hypoxia; A.U., arbitrary unit.
ANOVA, followed by Turkey’s multiple comparison test. Significance was defined as \( P < 0.05 \).

3. Results

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

We previously generated asbestos-induced rat MMs and analyzed them with expression microarray (GEO Accession No. GSE48298) [49]. Here re-analysis revealed that Ca9 was most highly expressed among all the CAs on the chip (Ca1, 7, 8, 9, 11, 12 and 15) in sarcomatoid subtype in comparison to epitheloid subtype, and was still significantly overexpressed in both the subtypes of MM (\( P < 0.01 \) and \( P < 0.05 \), respectively; Fig. 1A). Furthermore, Ca9 overexpression in MM, including all the three subtypes (epithelioid, biphasic and sarcomatoid subtypes), showed an inverse correlation (Pearson’s correlation, \( r = -0.52, P = 0.0095 \)) with survival (Fig. 1B). Thus, we decided to study Ca9. For further study, we selected two epithelioid (ACC-Meso-1 and NCI-H2052) and one sarcomatoid (NCI-H2373) human MM cell lines, together with one immortalized mesothelial cell line (MeT-5A).

Because CA9 is among highly expressed genes in response to hypoxia [35,50] and previous reports demonstrated that most solid tumors display low oxygen level (< 1.3% O2) [51], we evaluated CA9 expression by exposing MM cells in both hypoxic (1% O2 and 5% CO2 in N2 balanced atmosphere) and normal (5% CO2) culture conditions. Incubations were up to 48 h because MM cell proliferation under the hypoxia dose-dependently (40 and 75 \( \mu \)M) reduced migration of ACC-Meso-1 cells in response to CA9 inhibition. Cell proliferation was determined by measurement of cell viability (immediately) as a vehicle was used as a negative control [DMSO vehicle] unless indicated by bar). Refer to text for details.

![Fig. 2. CA9 inhibition antagonizes MM cell growth and migration under hypoxia. ACC-Meso-1 and MeT-5A cells were cultured under hypoxia with two distinct CA9-specific inhibitors, U104 [52] and S4 [53]. 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 antiproliferative effects (\( P < 0.001 \) and \( P < 0.01 \), respectively) on Met-5A cells though the effects were milder than in ACC-Meso-1 cells (Fig. 2B). As previous studies indicated a linkage between CA9 overexpression and metastasis in various cancers [54], we then evaluated the effect of CA9 inhibition on MM cell migration with wound healing assay. In contrast to vehicle treatment, S4 or U104 treatment under hypoxia dose-dependently (40 and 75 \( \mu \)M) reduced migration of ACC-Meso-1 cells at 24 h (Fig. 2C). These results suggest that CA9 activity is essential for MM cells to proliferate and migrate.
3.3. CA9 inhibition under hypoxia results in iron overload in lysosomes and mitochondria, inducing oxidative stress and triggering lipid peroxidation in MM cells

To study whether CA9 is associated with iron metabolism in MM cells under hypoxia, we evaluated the expression levels of iron-associated proteins after CA9 inhibition in MM cells under hypoxia. S4 or U104 treatment 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. (Figs. 3A and S2A).

Notably, these alterations were similar to the previous findings that abundant intracellular iron contributes to ferroptosis, a recently defined necroptosis-like regulated cell death with increased iron-uptake (TFRC) and decreasing iron storage (FTL/FTH) [55,56] and/or with NCOA4-mediated ferritin autophagy (ferritinophagy) [57]. Thus, we decided to determine levels and localization of catalytic Fe(II) in response to CA9 inhibition. S4 or U104 treatment in ACC-Meso-1 cells significantly increased the level of catalytic Fe(II) (P < 0.01 and P < 0.05, S4 and U104, respectively) (Fig. 3B), which was accompanied by oxidative stress (peroxides and mitochondrial O2−) and lipid peroxidation (Fig. 3C, D, 3E), surrogate markers for ferroptosis [56,58]. We also performed siRNA-induced CA9 knockdown in ACC-Meso-1 cells under hypoxia. Transfection of siRNA (20 nM) exhibited similar effects on the levels of iron metabolism-associated proteins as well as increased catalytic Fe(II) and lipid peroxidation (Figs. S2B, S2C, S2D). We then analyzed the distribution of catalytic Fe(II) with a confocal microscopy, which disclosed that overall catalytic Fe(II) signals were significantly increased with more localization in lysosomes (LysoTracker) and mitochondria (MitoTracker) (P < 0.001; N = 20) (Fig. 3F). These results suggest that CA9 plays a role in maintaining iron homeostasis in hypoxic MM cells.

3.4. CA9 inhibition under hypoxia leads to mitochondrial fission, contributing to autophagy in MM cells

Simultaneously with iron excess in mitochondria and lysosomes, we observed abnormality in mitochondrial morphology, displaying an increase in fragmented structure in response to S4 treatment (75 μM) in ACC-Meso-1 cells cultured under hypoxia (Figs. 3F and 4A). We measured the length of each mitochondrion stained with MitoTracker. S4-treated ACC-Meso-1 cells under hypoxia exhibited shortened (P < 0.001) mitochondrial length compared with the control group (Fig. 3G).
We also found increased \((P < 0.01 \text{ and } P < 0.05; \text{S4 and } \text{U104, respectively})\) transcription of \textit{DRP1}, encoding dynamin-related protein to catalyze the mitochondrial fission \cite{59}, after S4 (40 μM) or U104 (75 μM) treatment under hypoxia for 48 h (Fig. 4C). To further confirm this, we performed transmission electron microscopy. S4-treated ACC-Meso-1 cells presented diverse abnormalities in mitochondria, showing swelling, membrane rupture and fragmentation as well as engulfment by autophagic vacuoles (mitophagy) (Fig. 4D). A significant \((P < 0.001)\) increase in merged staining area (yellow) of Mito and LysoTracker was detected in S4-treated ACC-Meso-1 cells, which was rare in untreated control group under hypoxia for 48 h (Fig. 4E). Further confirmation of mitophagy was achieved by immunofluorescence staining of LC3B, a marker of autophagosome, and MitoTracker. S4-treated ACC-Meso-1 cells displayed a significantly \((P < 0.001)\) increased LC3B and MitoTracker merge staining (yellow) (Fig. 4F). In addition to LC3B, we also examined the protein level of LAMP-1, a marker for autophagosome and lysosome, by immunoblotting. Increased expression of both LAMP-1 and LC3B-II (lipidated form) were observed in a dose-dependent manner in response to S4 or U104 treatment in ACC-Meso-1 cells under hypoxia for 48 h (Fig. 4G).

3.5. CA9 inhibition induces both apoptosis and ferroptosis

S4 or U104 (75 μM) caused significant cell death detected by SYTOX Green at 48 h in ACC-Meso-1 cells under hypoxia (Fig. 5A). To define the mode of cell death induced by CA9 inhibition in MM cells, we first evaluated apoptotic markers. Both S4 and U104-treated ACC-Meso-1 cells showed a significant \((P < 0.05 \text{ and } P < 0.01; \text{S4 and } \text{U104, respectively})\) increase in late apoptotic cells (Annexin V-FITC+/PI+; shown as B+/+) under hypoxia (Fig. 5B)
revealed a significant \((P < 0.05)\) increase in early apoptotic cells (Annexin V-FITC\(^+\)/PI\(^-\)), shown as B+/-) whereas S4 did not. Confirmation of apoptosis was performed with TUNEL assay and validation of protein levels of cleaved caspase-3. Treatment with S4 or U104 (75 \(\mu\)M) not only induced TUNEL-positive cells with nuclear fragmentation but also increase in cleaved caspase-3 in ACC-Meso-1 cells cultured under hypoxia (Fig. 5C and D).

Because we observed increased catalytic Fe(II) with oxidative stress and lipid peroxidation (Fig. 3C–E) as well as mitophagy (Fig. 4EG) after CA9 suppression in MM cells, we speculated the involvement of ferroptosis. To study the association of ferroptosis, ferroptotic (Fer-1) and apoptotic (Z-VAD) inhibitors as well as an iron chelator (DFO) were cotreated with S4 or U104 in ACC-Meso-1 cells under hypoxia. Z-VAD (50 \(\mu\)M) treatment significantly \((P < 0.05\) and \(P < 0.01\), respectively) reversed CA9 inhibition-induced cell death by S4 (50 \(\mu\)M) or U104 (60 \(\mu\)M) treatment at 72 h (Fig. 5E). Fer-1 (3 \(\mu\)M) was significantly effective against S4 and U104 treatment \((P < 0.05\) and \(P < 0.01\), respectively). DFO at a low concentration (0.5 \(\mu\)M) significantly \((P < 0.01)\) attenuated S4-induced cytotoxic effect. Furthermore, we evaluated ferroptosis induced by a classical inducer, erastin, in ACC-Meso-1 cells and compared the levels of iron metabolism-associated proteins between those of CA9 inhibitors and of erastin. Erastin (20 \(\mu\)M)-induced MM cell death under hypoxia \((P < 0.001\) vs each control (CTRL) unless indicated by bar). Refer to text for details. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Excess iron has been associated with carcinogenesis through Fenton reaction catalyzed by Fe(II) \([8,10,60]\). Cancer may originate as side...
effects of using iron and oxygen for a long time [7, 61]. Carbonic anhydrases (CA; EC 4.2.1.1) are a superfamily (CA1-16) of metalloenzymes, equilibrating the reactions among CO2, bicarbonate and H+ [32], and thus are indispensable for intracellular pH regulation. Carbonic anhydrase 9 (CA9), a membrane-associated α-CA, has recently been a prognosis marker and also a drug target for various cancers [33]. In the present study we for the first time showed an intimate association of iron metabolism with hypoxia and redox regulation, where CA9 plays a central role at least in MM tumor cells, an iron excess-associated cancer [6]. Of note, this association was not observed in non-tumorous mesothelial cells (MeT-SA). Therefore, CA9 is an appropriate molecular target for the therapy of MM.

Cancer is now recognized as a disease of the genome, in which each cancer has its own evolutionary process through distinct mutation burdens [62]. Cancer presents characteristics of persistent proliferation (DNA replication and mitosis) and invasion, sometimes metastasis. Indeed, this is not easy, considering the coexistent hypoxia inside when the tumor becomes large with relative deficiency in vasculature and simultaneous need for abundant iron for DNA replication, starting with ribonucleotide reductase where iron is a cofactor [63]. Here we suggest, based on our results, that CA9 overexpression via HIF-1α activation works as a magic bullet for cancer: 1) CO2 elimination with regulation of intracellular pH for better cellular metabolism; 2) higher intracellular pH reduces soluble iron, including catalytic Fe(II) [60]; 3) hypoxia and extracellular acidity provide tumor microenvironment for selection over other kinds of non-tumorous cells under evolutionary pressure and proliferation [2], which may be efficiently performed at the outer plasma membrane by CA9. We have to be reminded that the first life on earth was born in the ancient sea of full of catalytic Fe(II), thus in a low pH [64]. In the present study we demonstrated that CA9 inhibition by specific inhibitors or by knocking down under hypoxia obstructs cellular proliferation and migration of MM, eventually leading to MM cell death.

Recently, fluorescent turn-on probes for catalytic Fe(II) have become available for time-lapse cell culture study or for frozen sections [46, 65–67], which provides precious information in situ. By the use of these probes, we found that catalytic Fe(II) is significantly increased with hypoxia in lysosomes, mitochondria and cytosol (Fig. S3A), which was further promoted by CA9 inhibition with enhancement in lysosomal and mitochondria (Fig. S3B). Interestingly, the alterations in expression of iron metabolism-associated genes were opposite in MM cells between hypoxia only and CA9 inhibition under hypoxia. Namely, hypoxia alone condition caused a logical response in iron metabolism to increased catalytic Fe(II) in MM cells. Namely, iron increment, presumably caused by increased solubility of iron according with the lowered pH, resulted in low iron intake and high iron storage to prevent iron toxicity. The results may be associated with the fact that efficiency of iron transporters, such as ZIP8/14 (SLC39A14) [68] and SLC11A2 [69], are pH-dependent. Still the MM cells tried to reserve iron intracellularly by decreasing FPN-1 [70].

However, in the case of CA9 inhibition with simultaneous hypoxia, MM cells responded to a situation of further increase in catalytic Fe(II) as if they lack iron, requiring more (seeking high iron intake and low storage with tight regulation). This is presumably due to a lethal condition, including critically increased lysosomal and mitochondrial catalytic Fe(II), resulting in oxidative stress and lipid peroxidation. Whereas how iron is transported across the mitochondrial outer membrane is not elucidated yet, the results indicate that CA9 at least partially controls iron metabolism in cells. Here the MM cells tried to purge catalytic Fe(II) outside by increased FPN-1. Autophagy can promote iron availability for tumor survival under hypoxia [71] and we believe that this mechanism was in motion. We observed on this condition numerous mitochondrial abnormalities, including fission, disruption and mitochondrial autophagy (mitophagy), consistent with other conditions involving oxidative stress or iron excess [72, 73]. Based on the results, we further analyzed the modes of MM cell death after CA9 inhibition under hypoxia, and found that this is a mixed cell death of apoptosis and ferroptosis [56] through autophagic process. Currently, there are twelve distinct modes of regulated cell death defined [74]. We recognized that three distinct modes of regulated cell death (autophagy, ferroptosis and apoptosis) were involved in the case of MM cell death by CA9 inhibition under hypoxia.

Limitations of our present work are: 1) specificities of the CA9 inhibitors are not complete [34], so we also performed knockdown experiments to confirm the results; 2) MM cells most probably evolved under excess iron environment during carcinogenesis [7], so further studies on other kinds of cancer are warranted to confirm that our results are universal for cancer.

In 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 [15]. Because CAs have been a drug target for various pathologies, including cancers, glaucoma, obesity and Alzheimer’s disease [34, 75], reexamination of other pathologies from the viewpoint of catalytic Fe(II) might be fruitful.

Conflicts of interest

The authors have no conflicts of interests to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101297.

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