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Inhibition of histone/lysine acetyltransferase activity kills $CoCl_2$-treated and hypoxia-exposed gastric cancer cells and reduces their invasiveness

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ABSTRACT

Hypoxia enhances immortality and metastatic properties of solid tumors. Deregulation of histone acetylation has been associated with several metastatic cancers but its effect on hypoxic responses of cancer cells is not known. This study aimed at understanding the effectiveness of the hydrazinocurcumin, CTK7A, an inhibitor of p300 lysine/histone acetyltransferase (KAT/HAT) activity, in inducing apoptosis of gastric cancer cells (GCCs) exposed to cobalt chloride ($CoCl_2$), a hypoxia-mimetic chemical, or 1% O_2. Here, we show that CTK7A-induced hydrogen peroxide (H_2O_2) generation in $CoCl_2$-exposed and invasive gastric cancer cells (GCCs) leads to p38 MAPK-mediated Noxa expression and thereafter, mitochondrial apoptotic events. Noxa induction in normal immortalized gastric epithelial cells after CTK7A and hypoxia-exposure is remarkably less in comparison to similarly-treated GCCs. Moreover, hypoxia-exposed GCCs, which have acquired invasive properties, become apoptotic after CTK7A treatment to a significantly higher extent than normoxic cells. Thus, we show the potential of CTK7A in sensitizing hypoxic and metastatic GCCs to apoptosis induction.

1. Introduction

Solid tumors are different from normal tissues in terms of their oxygen supply and consumption. The core of solid tumors become hypoxic due to inefficient blood supply (Karakashev and Reginato, 2015; Rankin and Giaccia, 2008). Hypoxia provides the ideal microenvironment for promoting metastasis (Sullivan and Graham, 2007). Naturally, like other solid tumors, gastric cancer malignancy and treatment resistance are largely, if not solely, determined by hypoxia (Vaupel and Mayer, 2007). Thus, selective therapeutic targeting of hypoxic metastatic gastric cancer cells is a challenging area of research.

Hypoxic cells undergo several hypoxia-adaptations. The master regulatory protein expressed in hypoxic cells is hypoxia-inducible

Abbreviations: Ac, acetylated; BH3, Bcl2 homology 3; CM-H_2-DCFDA, 5-(and 6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester; $CoCl_2 \cdot 6H_2O$, cobalt chloride hexahydrate; Cyt *c*, cytochrome *c*; DAPI, (4′,6-Diamidino-2-phenylindole Dilactate; ERK, extracellular signal-regulated kinases; GCC, gastric cancer cell; HAT, histone acetyltransferase; H_2O_2, hydrogen peroxide; Hif1, hypoxia-inducible factor 1; HRE, hypoxia-responsive element; IP, immunoprecipitation; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; SOD, superoxide dismutase; WT, wild type.

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factor1 (Hif1). Hif1 is a heterodimeric protein having two subunits, α and β. While Hif1α gets degraded in normoxia and hypoxia stabilizes it, Hif1β is constitutively expressed and its stability is not regulated by cellular oxygenation status (Huang et al., 1998). Hif1α dimerizes with Hif1β and transcriptionally activates a number of hypoxia-responsive genes by binding to the hypoxia-responsive elements (HREs) (Rohwer et al., 2009). p300 acts as a transcriptional coactivator of Hif1. p300 has an intrinsic histone or lysine (K) acetyltransferase (HAT or KAT) activity and functions as a protein scaffold (Chan and La Thangue, 2001; Santer et al., 2011). Autoacetylation induces acetyltransferase activity of p300. Both enhanced and suppressed (HAT) activity in tumor cells can lead to cell cycle arrest and apoptosis (Clarke et al., 1999; Kawamura et al., 2004). It is evident from the literature that the relation of HAT activity with cancer is tissue and context-dependent. Dysregulation of histone acetylation and deacetylation have been associated with gastric cancer progression and invasiveness (Yang et al., 2014). Studies have found expression of Hif1α in gastric cancer and have associated Hif1α with aggressive metastasis and treatment resistance (Liu et al., 2008; Rohwer and Cramer, 2010; Urano et al., 2006). Induction of apoptotic cell death in invasive cancer cells is a highly desired goal to achieve for cancer therapists. Cellular apoptosis is orchestrated by two separate signaling pathways- the mitochondria-mediated intrinsic pathway and the death receptor-mediated extrinsic pathway (Cotter, 2009). Chemo and radiotherapy induce the former pathway by involving the Bcl2 family proteins. A Bcl2 homology 3 (BH3)-only tumor suppressor protein Noxa is transcriptionally induced by Hif1 and induces mitochondria mediated intrinsic apoptotic pathway (Gomez-Bougie et al., 2011).

In this study, we investigated the role of p300 HAT activity on Noxa-mediated apoptosis in $CoCl_2$-treated and 1% O_2-exposed GCCs. We showed that downregulation of HAT activity significantly induced reactive oxygen species (ROS) generation as well as Noxa-mediated apoptosis selectively in hypoxic GCCs but not in non-hypoxic GCCs. In addition, the expression of metastatic markers in $CoCl_2$-treated GCCs was also downregulated after suppression of HAT activity by the hydrazinocurcumin, CTK7A. Thus, this study revealed a previously undescribed mechanism for how CTK7A can induce apoptosis in hypoxia-exposed invasive GCCs and further enriched our understanding of its antitumor effects.

2. Methods

2.1. Cell lines

GCCs AGS, MKN 45, KATO III were cultured and maintained as previously described (Bhattacharyya et al., 2009). Immortalized human GCC cell HFE145 and pDsRed2 (Clontech, CA, USA)-expressing AGS stable cells were maintained in 10% heat-inactivated FBS-supplemented RPMI 1640. Most of the studies were performed using non-metastatic AGS cells so that metastasis induction could be clearly identified. HFE145 cells were used to study properties of non-malignant human gastric epithelial cells. *hif1α* knockdown cells were prepared as described previously (Rath et al., 2015).

2.2. Chemicals and reagents

Cobalt chloride hexahydrate ($CoCl_2{\cdot}6H_2O$) (Sigma-Aldrich, MO, USA) or 1% O_2 was used to induce hypoxia in AGS cells as per standard methods (Rath et al., 2016; Wu and Yotnda, 2011). CTK7A at 100 μM dose (Arif et al., 2010) either alone or in combination with $CoCl_2$ (200 μM) was used for 24 h treatment. ERK1/2 inhibitor PD98059, p38 MAPK inhibitor SB203580, and JNK inhibitor II SP600125 (all from Calbiochem, CA, USA) were used at 25 μM dose for 1 h prior to $CoCl_2$ and CTK7A treatment. Superoxide dismutase (SOD) and catalase (CAT) (both from Sigma Aldrich) were used at 200 units/ml and 350 units/ml dose, respectively, for 4 h prior to treatment with $CoCl_2$ and CTK7A. 5-(and 6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2-DCFDA; Invitrogen, CA, USA) was used to detect intracellular ROS generation.

2.3. Whole cell, nuclear, cytosolic and mitochondrial lysate preparation

Whole cell lysates were prepared by standard protein isolation protocol. Nuclear and cytoplasmic fractions were isolated using NE-PER Kit following manufacturer's instruction (Thermo Scientific, IL, USA). Mitochondrial lysates were prepared following a previously-described protocol (Rath et al., 2015).

2.4. Western blots, antibodies and immunoprecipitation

Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting. Membranes were probed with specific antibodies for p300, acetylated lysine, Hif1α, Noxa, Twist1 (Abcam, MA, USA), caspase 3, caspase 9, Cytochrome *c*, N cadherin, E cadherin, phospho p38 MAPK, phospho ERK and phospho JNK (Cell Signaling Technology, MA, USA). GAPDH (Imgenex Corporation, CA, USA) and Cox IV (Cell Signaling Technology, MA, USA) antibodies were used as loading controls for the cytosolic fraction and mitochondrial fractions, respectively. Proteins were detected by using Super Signal West Femto kit (Thermo scientific). Images were taken with Chemidoc XRS (Bio-Rad Laboratories, CA, USA) equipped with Quantity One-4.6.9 software.

2.5. Flow cytometry

Apoptosis was quantified by flow cytometry. AGS cells were treated with $CoCl_2$ (Sigma-Aldrich) alone and/or CTK7A for 24 h or left untreated. Cell pellets were washed twice with chilled PBS and stained with Annexin V PE/7-AAD (BD Biosciences, CA, USA) according to manufacturer's instruction. 1×10^4 cells were acquired per sample using FACSCalibur Flow Cytometer (BD Biosciences). Results were analyzed by CellQuest Pro software (BD Biosciences).

2.6. Confocal microscopy

pDsRed2 (Clontech)-expressing AGS stable cells were used for confocal microscopy. These cells were treated with $CoCl_2$ and/or CTK7A or left untreated for 24 h. Noxa translocation to mitochondria and cytochrome *c* release from mitochondria were studied in the above experimental condition. Cells were fixed with 4% paraformaldehyde at 37 °C for 15 min followed by incubation with DAPI (4′, 6-Diamidino-2-Phenylindole, Dilactate (Invitrogen) for 20 min. Mitochondrial morphology was studied as described earlier (Rath et al., 2015). Fragmentation of mitochondria, as assessed by roundness, circularity and length, was measured by ImageJ software (NIH, MD, USA). Roundness $[4 \times (\text{surface area})/(\pi \times \text{major axis}^2)]$ and circularity $[4\pi \times (\text{surface area}/\text{perimeter}^2)]$ together represented mitochondrial sphericity (sphericity value 1 = perfect spheroid).

2.7. Transwell migration and invasion assay

Cell migration and invasion assays were performed using 8-μm pore size Transwell Biocoat control inserts (migration assay)