Food and Chemical Toxicology 106 (2017) 260–272

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Metformin and caffeic acid regulate metabolic reprogramming in human cervical carcinoma SiHa/HTB-35 cells and augment anticancer activity of Cisplatin via cell cycle regulation

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ARTICLE INFO

Article history:
Received 13 April 2017
Received in revised form
25 May 2017
Accepted 28 May 2017
Available online 30 May 2017

Keywords:
Metformin
Cisplatin
Caffeic Acid
Cancer
Metabolic reprogramming
Apoptosis

ABSTRACT

Metformin shows benefits in anticancer prevention in humans. In this study, normal human fibroblasts (FB) and metastatic cervical cancer cells (SiHa) were exposed to 10 mM Metformin (Met), 100 μM Caffeic Acid (trans-3,4-dihydroxycinnamic acid, CA) or combination of the compounds. Both drugs were selectively toxic towards cancer cells, but neither Met nor CA treatment suppressed growth of normal cells. Met and CA regulated metabolic reprogramming in SiHa tumor cells through different mechanisms: Met suppressed regulatory enzymes Glurtaminase (GLS) and Malic Enzyme 1 (ME1) and enhanced pyruvate oxidation via tricarboxylic acids (TCA) cycle, while CA acted as glycolytic inhibitor. Met/CA treatment impaired expression of Sterol Regulatory Element-Binding Protein 1 (SREBP1c) which resulted in alleviation of de novo synthesis of unsaturated fatty acid. The toxic action of CisPt was supported by Met and CA not only in tumor cells, but also during co-culture of SiHa GFP+ cells with fibroblasts. Furthermore, Met and CA augmented Cisplatin (CisPt) action against quiescent tumor cells involving reprogramming of cell cycle. Our findings provide new insights into specific targeting of mitochondrial metabolism in neoplastic cells and into designing new cisplatin-based selective strategies for treating cervical cancer in humans with regard to the role of tumor microenvironment.

1. Introduction

To date, treatment protocols in cervical malignancy have been based on the application of Cisplatin (*Cis*-diamminedichloroplatinum(II), *Cis*-Pt) alone or as combined treatment with another cytostatic drug, 5-fluorouracil. However, the application of conventional therapies is associated with substantial toxicity to whole organism and may lead to the development of resistance to drugs (Barbera and Thomas, 2009). Hence, more specific interventions are highly required (Li et al., 2015). Growing evidence indicate that targeting metabolic reprogramming of neoplastic cells may be effective anticancer strategy (Lopez and Banerji, 2017). Despite intensive study, the metabolic regulation of tumor cells have not been fully elucidated. However, several molecular mechanisms were pointed out to drive metabolic adaptation in cancer cells, including enhanced production of intermediates via mitochondrial tricarboxylic acids (TCA) cycle. It was proposed that anti-cancer intervention may aim at increased glycolysis and glutamine addiction which are the main features of malignant metabolism (Erickson and Cerione, 2010). At the same time, tumors may reveal higher sensitivity to drugs that disrupt energy homeostasis, such as Metformin (1,1-dimethylbiguanide, Met) (Bost et al., 2016). In particular, the fact that mitochondrial metabolism of tumor cell may substantially differ from normal cell may be useful for designing therapies more specific towards malignancy (Ganapathy-Kanniappan and Geschwind, 2013).

Metformin is a widely administered, safe and effective drug to treat hyperglycaemia in patients with type 2 diabetes. Several case-control studies reported beneficial effects of Met on cancer mortality in humans. Metformin treatment was found to be associated with a reduction of risk for cancer incidence in general (Malek et al., 2013); however, it can directly protect against specific cancers (Sacco et al., 2016), as shown in clinical trials for breast and

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http://dx.doi.org/10.1016/j.fct.2017.05.065
0278-6915/

endometrial malignancy (Guo et al., 2016; Tang et al., 2017), liver (Cauchy et al., 2017), colorectal, pancreas and lung cancers (Martinez-Outschoorn et al., 2017). Mitochondrial metabolism which plays central role in tumorigenesis was proposed to be crucial intracellular target of Met (Cairns et al., 2011; Sacco et al., 2016).

Taking into consideration molecular advantages easily developed by malignant cells, downregulation of one particular molecular target or even pathway may be not sufficient to suppress cancer growth. Many recent reports rather suggest addressing of multi-targeted approach and using a combination of agents regarding different toxic mechanisms, specific towards certain cancer cells (Lopez and Banerji, 2017; Forbes-Hernández et al., 2014). Metformin and natural antioxidant Caffeic Acid (*trans*-3,4-dihydroxycinnamic acid, CA) have been reported before to modulate particular intracellular targets and present synergistic and/or additive action when combined with other anticancer approaches, as shown under rigorous research (Qi et al., 2016; Lee et al., 2011). In line with these findings, in our previous work we showed that Met in combination with CA alleviated survival of cervical cancer HTB-34 cells derived from secondary tumor via regulation of mitochondrial metabolism (Tyszka-Czochara et al., 2017). In present study, we aimed to explore the action of the drugs on metabolism and survival of metastatic human cervical cancer cells (SiHa). In order to assess if metabolic alterations exerted by tested compounds might be specific to neoplastic cells, we also employed normal human fibroblasts (FB) as a normal cell line reference. Recently, a combination strategy that uses factors sensitizing neoplastic cells to the action of chemotherapeutic drugs has been proposed and considered as more effective in tumor cell elimination in cervical carcinoma (Martinez-Outschoorn et al., 2017; Umar et al., 2012). Based on that concept we tested if Met and/or CA may significantly support the suppressive effect of *Cis*-PT against metastatic cells.

Hanahan and Wienberg (2011) well discussed the contribution of stromal cells to invasion and metastasis of tumor cells and related mechanisms that promote cancer progression. Emerging understanding of interaction of cancer cells with normal cells of adjacent tissues lead to the conclusion that effective therapeutic intervention has to address the possible interactions of cancer cells with cells of surrounding tissues (Barar and Omidi, 2013). In fact, tumor microenvironment has been found to elicit metastasis under specific circumstances. The reverse Warburg effect plays a major role in metabolic alterations, described previously in human breast cancers by Pavlides et al. (2009). Malignant cells may control the metabolism of surrounding fibroblasts and even recruit stromal cells to support their growth by providing lactate as additional carbon source for malignant metabolism (Romero et al., 2015; Cairns et al., 2011). What is more, the outcome of anti-cancer treatment may change dramatically under the influence of stroma. Therefore, in order to clarify how stromal cells interact with cervical tumor cells under exposition to Met and CA, we tested the influence of the compounds and CisPt on survival of SiHa *GFP*$^+$ cells grown in co-cultures with normal human fibroblasts.

2. Materials and methods

2.1. Cell culture of human cervical cancer cells (SiHa) and human fibroblasts (FB)

Human cervical carcinoma HTB-35 cells (ATCC designation HTB-35, SiHa) and human fibroblasts, FB (ATCC designation CRL 2522) were derived from the American Type Cell Culture collection (LGC Standards-ATCC, Teddington, Great Britain). SiHa cells were maintained as a monolayer culture in DMEM - Dulbecco's Modified Eagle's and FB were grown in EMEM - Medium Eagle's Minimum Essential Medium (Lonza, Walkersville, MD, USA), both supplemented with 10% FBS (Eurex Sp z o.o., Gdansk, Poland) and 50 μg/ml of gentamicin, at 37 °C in a humidified atmosphere of 5% CO_2. (Nunes et al., 2014). For experiments, SiHa cells at a density of 1.0×10^5 cells/mL and FB at a density of 1.5×10^5 cells/mL were placed in cell culture plates (Sarstedt, Numbrecht, Germany) and incubated to reach adequate confluency. Then cells were kept for 24 h in EMEM-containing antibiotic and 0.5% of bovine serum albumin (BSA, Sigma-Aldrich, Seelze, Germany). Then the medium in each well was replaced with a new one with adequate volumes of stock solution of CA (100 μM, Sigma-Aldrich), Met (10 mM, Sigma-Aldrich) or both chemicals and exposition of cells was continued for 24 h. Cisplatin (*Cis*-Pt, Sigma-Aldrich) was dissolved in DMSO and the stock solutions were added directly to the culture media at final concentration 20 μM. After incubations, cells and media were collected. The number of cells was assessed by automatic cell counter Countess (Gibco Laboratories, Gaithersburg, MD, USA) and the morphology of cell culture was investigated by an inverted light microscope (Olympus IX-70 microscope with fluorescence, Olympus, Hamburg, Germany).

2.1.1. Preparation of SiHa GFP$^+$cells

Lentiviral particles were generated accordingly to manufacturer's protocol. Briefly, the established amount of HEK293T cells (ATCC: CRL 3216) was seeded on culture flask in DMEM medium containing 10% of FBS (Eurex). The transfection mixture contained the established amounts of plasmid DNA and the transfection mix was transferred into fresh culture medium containing 25 μM chloroquine (Sigma-Aldrich). The medium containing lentiviral particles was harvested, centrifuged, filtered and used in next step to generate virus introducing Green Fluorescent Protein (GFP) into SiHa cells. To determine the titer of lentiviral stocks, HT1080 cells (ATCC: CCL-121) were infected. Four hours prior to transduction cells were seeded on plates and various volumes of harvested media were added to cells. 48 h after transduction the percentages of modified (GFP-expressing, GFP$^+$) cells were assessed by cytometer (FACSCanto10C, BD Biosciences Immunocytometry Systems, San Jose, CA, USA) to calculate Transduction Units (TU) in 1 ml of each harvested medium. To obtain SiHa modified cell line, SiHa wild-type cells were infected using lentiviral particles introducing GFP gene generated in the previous step. Transduction was performed with MOI = 5 (Multiplicity of Infection) and 6 μg/ml Polybrene. SiHa *GFP*$^+$ cell line was purified using FACS Aria II cell sorter (BD, Franklin Lakes, NJ, USA). The percentage of green fluorescent cells in modified cell line was assessed using flow cytometer and the population of 80% and more of *GFP* + cells was used in experiments.

2.1.2. Co-culture of SiHa GFP$^+$ cells and FB

The exponentially dividing SiHa *GFP*$^+$ cells and FB were trypsynized and counted by automatic cell counter (Gibco). Then the mixture of cells was prepared at ratio of 3 FB to 1 SiHa *GFP*$^+$ in EMEM medium (Lonza) and seeded into wells at a density of 1.5×10^5 cells/mL. The cells were grown for next 24 h and co-cultures were inspected with light and fluorescence microscope to evaluate the number and the morphology of SiHa *GFP*$^+$ cells and FB. The percentages of GFP-expressing SiHa cells in co-culture were assessed by flow cytometer and then co-cultures were incubated with tested compounds as described above.

2.2. Viability and cytototoxicity assays

Cell viability was assessed using MTT assay, as described previously (Adach et al., 2015). MTT, 3-[4,5-dimethylthiazol-2yl]-2,5-