Journal of Ethnopharmacology 203 (2017) 47–54

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Anemone rivularis inhibits pyruvate dehydrogenase kinase activity and tumor growth

Tae-Wook Chung[a], Jung Hee Lee[a], Hee-Jung Choi[a], Mi-Ju Park[a], Eun-Yeong Kim[a], Jung Ho Han[a], Se Bok Jang[b], Syng-Ook Lee[c], Sang Woo Lee[d], Jin Hang[e], Li Wan Yi[e], Ki-Tae Ha[a,*]

[a] *Department of Korean Medical Science, School of Korean Medicine and Healthy Aging Korean Medical Research Center, Pusan National University, Yangsan, Gyeongnam, Republic of Korea*
[b] *Department of Molecular Biology, College of Natural Science, Pusan National University, Busan, Republic of Korea*
[c] *Department of Food Science and Technology, Keimyung University, Daegu, Republic of Korea*
[d] *Korea Research Institute of Bioscience and Biotechnology, Ochang, Chungbuk 34141, Republic of Korea*
[e] *Yunnan Academy of Agricultural Sciences, Yunnan 650223, China*

ARTICLE INFO

Keywords:
Warburg's effect
Pyruvate dehydrogenase kinase
Anemone rivularis
Mitochondria
Apoptosis

ABSTRACT

Ethnopharmacological relevance: *Anemone rivularis* Buch.-Ham. ex DC. (Ranunculaceae) have been used as a traditional remedy for treatment of inflammation and cancer. However, there is no report demonstrating experimental evidence on anti-tumor action of *A. rivularis*.

Aim of study: The Warburg's effect, preference of aerobic glycolysis rather than oxidative phosphorylation (OXPHOS) even in oxygen rich condition, is focused as one of major characteristics of malignant tumor. Thus, we investigated the effect of *A. rivularis* on the Pyruvate dehydrogenase (PDH) kinases (PDHKs), a major molecular targets for reducing aerobic glycolysis.

Materials and methods: The ethanol extract of whole plant of *A. rivularis* (ARE), fingerprinted by high performance liquid chromatography (HPLC), was applied to *in vitro* and cell-based PDHK activity assays. The effect of ARE on cell viabilities of several tumor cells was estimated by MTT assay. The expression of phosphor-PDH, PDH and PDHK1 were measured by Western blot analysis. The production of reactive oxygen species (ROS) and apoptosis was measured by fluorescence-activated cell sorting analysis, using 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) and Annexin V/propidium iodide (PI) staining, respectively. Mitochondrial membrane potential was examined by tetramethylrhodamine methyl ester (TMRM) staining. *In vivo* anti-tumor efficacy of ARE was estimated by means of tumor volume and weight using allograft injection of murine Lewis lung carcinoma (LLC) cells to dorsa of C57BL/6 mice.

Results: ARE inhibited the viabilities of several cancer cells, including MDA-MB321, K562, HT29, Hep3B, DLD-1, and LLC. ARE suppressed PDHK activity in *in vitro* kinase assay, and also inhibited aerobic glycolysis by reducing phosphorylation of PDHA in human DLD-1 colon cancer and murine LLC cells. The expression of PDHK1, a major isoform of PDHKs in cancer, was not affected by ARE treatment. Moreover, ARE increased the both ROS production and mitochondrial damage. In addition, ARE suppressed the *in vitro* tumor growth through mitochondria-mediated apoptosis. The growth rates of allograft LLC cells were also reduced by ARE treatment.

Conclusions: Here, we firstly report that ARE inhibits PDHK activity and growth of tumor in both *in vitro* and *in vivo* experiments. Therefore, we suggest ARE as a potential candidate for developing anti-cancer drugs.

1. Introduction

The preference of glycolysis for ATP production rather than oxidative phosphorylation (OXPHOS) even in the presence of oxygen is a characteristic of most cancer cells (Batra et al., 2013). The aerobic glycolysis, also termed as Warburg's effect, offers several advantages to malignant cells, including increased lactate production, subsequent acidification of tumor environment, activation of pro-survival signaling, and preservation of carbon sources (Gatenby and Gillies, 2004; Vander Heiden et al., 2009). In recent studies, aerobic glycolysis is accepted as therapeutic target for tumor treatment. Therefore, the key enzymes involved in Warburg's effect, such as hexokinase, pyruvate

* Correspondence to: Healthy Aging Korean Medical Research Center, Pusan National University, Busandaehak-ro 49, Yangsan, Gyeongsangnam-do 50612, Republic of Korea.
E-mail address: hagis@pusan.ac.kr (K.-T. Ha).

http://dx.doi.org/10.1016/j.jep.2017.03.034
Received 4 November 2016; Received in revised form 19 March 2017; Accepted 20 March 2017
Available online 21 March 2017
0378-8741/

kinase, lactate dehydrogenase (LDH), and PDHK, are emerging target for developing novel anti-cancer drugs (Avantaggiati, 2013; Batra et al., 2013).

Among these enzymes, PDHK is a mitochondrial enzyme reducing pyruvate utilization by phosphorylating E1α subunit (PDHA) of pyruvate dehydrogenase complex (PDC), a complex of enzymes that converts pyruvate to acetyl-CoA (Sutendra and Michelakis, 2013; Zhang et al., 2014). Four isoforms of PDHKs bind to the E2 subunit of PDC and phosphorylate PDHA at three serine sites, Ser232, Ser293, and Ser300 (Rardin et al., 2009; Sutendra and Michelakis, 2013). In many malignant tumors, especially in hypoxic condition, PDHK1 acts as a major enzyme that phosphorylates PDHA and inactivates mitochondrial PDC (Hitosugi et al., 2011; Kim et al., 2006). Inhibition of PDHK1 with siRNAs or dichloroacetate (DCA) shifts the metabolism of tumor cells from glycolysis to OXPHOS and reduces the resistance to mitochondria-mediated apoptosis (Jeoung, 2015; Sutendra and Michelakis, 2013). Therefore, PDHKs inhibitors including DCA, AZD7545, radicicol, and JX06, are regarded as the promising candidates for cancer inhibition (Jeoung, 2015; Tso et al., 2014).

Anemone rivularis Buch.-Ham. ex DC. (belong to Ranunculaceae) is a perennial plant widely distributed in China, Nepal, India, and Vietnam (Mizutani et al., 1984; Rajbhandari et al., 2009). The plant was used as a traditional remedy to treat inflammatory diseases, respiratory disease, and cancer (Mizutani et al., 1984; Rajbhandari et al., 2009; Zhao et al., 2012). Several previous studies revealed the phytochemical constituents (Chau et al., 2012; Liao et al., 2001; Mizutani et al., 1984; Zhao et al., 2012) and its pharmacological activities (Rajbhandari et al., 2009; Wang et al., 2014b). However, there is no preceding report demonstrating anti-tumor action of *A. rivularis*.

In this study, we demonstrated that ethanol extract of *A. rivularis* has an inhibitory effect on PDHK activity and induces apoptosis of cancer cells through mitochondrial pathway. Within our best knowledge, it is the first report providing the experimental evidence of anti-tumor activity of *A. rivularis* and its mode of action.

2. Materials and methods

2.1. Materials

Antibodies against caspase-3, caspase-9, and poly ADP-ribose polymerase (PARP) were purchased from Cell Signaling Technology. Antibody for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was supplied from Santa Cruz Biotechnology. Antibodies for PDHK1 were provided by Enzo Life Sciences. PDHA antibody was purchased from Invitrogen. Antibody for phospho-PDHA was supplied from Calbiochem, and all reagents including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich unless indicated otherwise.

2.2. Preparation of plant materials

Ethanol extract of whole plant of *A. rivularis* was supplied by International Biological Material Research Center (no. FBM059-043; Daejeon, Korea). The plant was collected in Yunnan Province of China in 2008 and authenticated by the Chief of Institute of Medicinal Plants (IMP), Jin Hang (Yunnan Academy of Agricultural Sciences; YAAS). A voucher specimen is kept at the herbarium of YAAS. Briefly, the dried and refined whole plant of *A. rivularis* (100 g) was extracted with 800 mL of 95% ethanol for 2 h, twice. The extract was percolated with filter paper (3 mm; Whatman PLC, Kent, UK), condensed using a rotary evaporator (Buchi, Swiss), and lypophilized using a freeze dryer (Christ, Germany). The powder (8 g; yield 8%; abbreviated as ARE) was dissolved in distilled water for stock solution (20.56 ± 0.09 mg/mL) and diluted with culture medium (for *in vitro* assay) or phosphate buffered saline (PBS; for *in vivo* treatment) before used in the experiments.

2.3. High-performance liquid chromatography (HPLC) analysis of plant material

Phytochemical feature of ARE was verified by HPLC analysis. HPLC analysis was conducted with an Agilent 1200 series system (Agilent Technologies). KR100-5C18 column (AkzoNobel, Netherland; 4.6×250 mm; pore size, 3.5 μm) was used as analytical column. LC solution software (version 1.24) was used for data analysis. The mobile phases were solvent A [acetonitrile] and solvent B [0.1% trifluroacetic acid aqueous (v/v)] and solvent B (acetonitrile). The flow was as follows: (A)/(B)=96/4. The analysis was performed at 70 °C of column temperature and at a flow rate of 1 mL/min with detection at 205 nm. The injection volume to column was 10 μL. A standard solution containing huzhangoside A (Chau et al., 2012) was prepared as dissolved in distilled water.

2.4. Cell culture

Human colon cancer HT29 cells and DLD-1 cells, and murine Lewis lung carcinoma (LLC) cells were provided by the American Type Culture Collection (ATCC, VA, USA). Human breast cancer MDA-MB231 cells, human hepatocellular carcinoma Hep3B cells, and human chronic myelogenous leukemia K562 cells were supplied by Korean Cell Line Bank (Seoul, Korea). The K562 cells were grown in RPMI 1640 medium (HyClone™, GE Healthcare Life Sciences, UT, USA) and the other cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Welgene, Gyeongsan, Korea) supplemented with *L*-glutamine (200 mg/L), 10% (v/v) heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, MO, USA), and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; Thermo Fisher Scientific, MA, USA). The cells were cultured in a humidified CO_2 incubator at 37 °C, 5% CO_2 condition before the experiments.

2.5. Cell viability assay

The cytotoxicity of ARE was examined using a MTT assay. Briefly, the cells were incubated in 24-well culture plates in the indicated concentrations of ARE for 24 h. MTT solution (2.0 mg/mL) was added to each well of plates and the culture plates were cultured for 4 h at 37 °C and 5% CO_2 conditions. In suspended cell case for K562 cells, after incubation, the culture plates were centrifuged at 3000 rpm for 10 min. The culture media were removed and formazan crystals made in living cells were measured by the absorbance at 540 nm with a Spectramax M2 microplate reader (Molecular Devices).

2.6. *In vitro* pyruvate dehydrogenase kinase (PDHK) assay

The kinase activity of PDHK was performed as described previously (Hitosugi et al., 2011). Briefly, GST-PDHK1 construct (obtained from Jing Chen, Emory University) was transfected into 293T cells using polyethylenimine (PEI, Sigma-Aldrich). At 24 h after transfection, cells were harvested and lysed using buffer [150 mM NaCl, 10 mM HEPES (pH 7.45), 1% NP-40, 5 mM NaPyrophosphate, 5 mM NaF, 2 mM Na_3VO_4]. GST-PDHK1 was pulled down by Glutathione Sepharose 4B beads (Amersham Bioscience). The beads bound with GST-PDHK1 were washed with PBS, followed by application to PDHK1 kinase assay. The beads were incubated with 50 ng of recombinant PDHA1 (PDHA, E1p clone obtained from David Chuang, University of Texas) as a substrate of PDHK1 for 5 min at 30 °C in PDHK1 kinase buffer (20 mM potassium phosphate buffer pH 7.5, 0.1 mM EDTA, 1 mM $MgCl_2$, 2 mM DTT and 250 μM ATP). The samples were applied to SDS-PAGE, followed by immunoblotting using GST, PDHA1 and phospho-PDHA1 (Ser293) antibodies.