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The mitochondrial dysfunction plays an important role Ćin urethane-induced lung carcinogenesis

Gangjun Du *,[1], Ting Sun [1], Yaping Zhang, Haihong Lin, Jiahuan Li, Weijie Liu, Yingying Wang, Bei Zhao, Hongru Li, Yinghui Liu

Institute of Pharmacy, Pharmacy College of Henan University, Jinming District, Kaifeng, Henan 475004, China

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ABSTRACT

Mitochondrial dysfunction is an important factor as genetic change in controlling the growth of tumors. The current study explored whether mitochondrial dysfunction correlated with urethane-induced lung carcinogenesis in BALB/c and C57BL/6 mice that were given single- or multi-dose intraperitoneal injections of urethane. We found that mice susceptible to lung tumor formation displayed a rapid increase in the respiratory control ratio in lung mitochondria after urethane exposure, whereas resistant mice that failed to develop lung tumors maintained the same respiratory control ratio as normal, untreated mice. Furthermore, repeated urethane administration or continuous 2,4-dinitrophenol (an uncoupling agent of oxidative phosphorylation) treatment, following a single urethane exposure, could overcome resistance to carcinogenesis. In contrast, multi-dose urethane-treated mice that received genipin (a highly selective inhibitor of uncoupling protein 2) following their first dose of urethane showed a lower tumor incidence. In addition, a higher uncoupling protein 2 level and a lower complex IV level in the lungs correlated with subsequent tumor formation in BALB/c and C57BL/6 mice. In vitro, urethane suppressed cell proliferation and induced soft-agar colonies in parental L929 cells with complete mitochondrial DNA but not in ρ° L929 cells lacking mitochondrial DNA. These studies suggest that abrogation of mitochondrial is essential during urethane induced lung carcinogenesis and that uncoupling inhibition can reverse cancer morphogenesis, thus presenting an appealing method to prevent lung carcinogenesis.

1. Introduction

The 5-year survival rate for lung cancer remains poor despite aggressive medical therapy (Siegel et al., 2012). Over the past several decades, it has become increasingly clear that altered metabolism is a key aberrant biological difference between tumor cells and normal differentiated cells (Yuneva et al., 2012). Cancer cells depend mainly on aerobic glycolysis, defined as the aerobic breakdown of glucose into the energy-storing molecules of adenosine triphosphate (ATP) (de-Souza et al., 2011). Otto Warburg noted that cancer cells had an irreversible injury to respiration that lead to decreased oxidative phosphorylation and increased aerobic glycolysis, despite the presence of enough oxygen for aerobic respiration (Romero-Garcia et al., 2011). The existing evidence suggests that mitochondrial dysfunction plays an important role in supporting the malignant behavior of cancer cells (Redente et al., 2007). Recent studies have shown that mutations in mitochondrial (mtDNA) or changes in mtDNA content also underlie the irreversible injury to respiration (Yang et al., 2010). However, whether OXPHOS deficiency correlates with lung carcinogenesis is currently unknown.

Mouse models for lung cancer can serve as a valuable tool not only for understanding basic lung tumor biology but also for the development and validation of new tumor intervention strategies (Martinez-Outschoorn et al., 2011). Urethane is a recognized genotoxic carcinogen, with widespread occurrence in fermented foods and beverages (Battaglia et al., 1990). In 2007, the international agency for research on cancer (IARC) upgraded its classification of urethane to group 2 A that is probably carcinogenic to humans (Lachenmeier et al., 2010). In mice, urethane-induced pulmonary adenomas have been extensively used as a model to study lung tumors and to provide insights into the molecular events involved in carcinogenesis (Calbo et al., 2011). Several studies have identified similarities in the morphology and molecular characteristics between urethane-induced mouse lung tumors and human lung adenocarcinomas, further supporting the utility of a mouse model (Bauer et al., 2011). Because of the lack of success in lung cancer chemoprevention and chemotherapy, it is important to consider the treatment of existing

* Corresponding author. Tel./fax: +86 378 3880680.
E-mail address: dugangjun@henu.edu.cn (G. Du).
[1] These authors contributed equally to this work.

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preinvasive neoplastic lesions because large numbers of individuals have previous exposure to carcinogens (Ueno et al., 2012). A deeper understanding of the complex mechanisms implicated in carcinogenesis may aid in developing strategies to reduce morbidity and mortality from cancer. We proposed that mitochondrial dysfunction also plays an important role in urethane-induced lung carcinogenesis, we observed the relative contributions of mitochondrial dysfunction on carcinogenesis with the goal of (a) identifying the early changes in cancer progression and (b) developing new therapeutic strategies that tackle the mitochondria to prevent lung cancer.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) was from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS), Penicillin, and Streptomycin were from Gibco BRL (Rockville, MD). Sodium pyruvate, Ethidium bromide, Uridine, MTT(3-(4,5-dimethyl-2-thiazole)-2,5 -diphenyltetrazolium bromide), Succinate, ADP, Glutamate, Malate 2,4-Dinitrophenol, Glucose–Hexokinase, Glucose-6-Phosphotate dehydrogenase, Rh123, and NADP were purchased from Sigma. Polyclonal anti-uncoupling protein-2 (UCP2), Polyclonal anti-complex IV subunit 1 and 2′,7′-dichlorodihydrofluorescein-diacetate (DCHF-DA) were purchased from Molecular Probes, Inc. Complex I, Complex II, Complex III and Complex IV activity kits were obtained from GenMed (Eugene, OR). Luciferin–Luciferase was purchased from Merck Biosciences. Genipin was obtained from Shanxi Huike Botanical Development Co. (China). A secondary antibody (sheep anti-mouse IgG horseradish peroxidase-linked antibody) was purchased from Sino-American Biotechnology Co., Ltd (China). Agarose, Promega (USA). All other reagents were of analytical grade from commercial sources.

2.2. Animals

The female BALB/c and C57BL/6 mice, 5–7 weeks of age, were obtained from Beijing Weitonglihua Animal Co. The mice were maintained in a pathogen-free animal facility for at least 1 week prior to experiment. Mice were housed under controlled conditions (22 ± 2 °C, 65 ± 15% relative humidity, air exchange rate 15 times/h, 12 h light/dark cycles) in filter top cages and received a standard pellet diet and tap water ad libitum during the study. The animal use committee of the Pharmaceutical College of Henan University approved all animal study protocols described in this study, and experiments were conducted in compliance with the guide for the care and use of laboratory animals.

2.3. Cell culture

Mouse embryonic lung fibroblast (L929) cell lines were purchased from American type culture collection (ATCC; Manassas, VA). ρ° L929 cells lacking mitochondrial DNA were generated by treatment of parental L929 cells with 50 ng/ml ethidium bromide in growth medium supplemented with 50 μg/ml uridine and 110 μg/ml sodium pyruvate according to the previous procedures (Liu et al., 2005). The medium was changed every 2 days, and cell-based functional assays were carried out after one month of successive subcultivation. The depletion of mitochondrial DNA was confirmed by PCR amplification and culture of uridine defect (data not shown). L929 cells were cultured in a 4.5 g/l glucose DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mmol/L L-glutamine and 110 μg/ml sodium pyruvate. The ρ° L929 cells were cultured using the same medium plus 50 μg/ml uridine, which provides an alternative source of energy through glycolysis to ensure optimal growth. All cell cultures were grown in a humidified incubator at 37 °C with 5% CO_2.

2.4. Cell proliferation assay

L929 cells and ρ° L929 cells at 10,000 cells per well were seeded in a 96-well plate overnight at 37 °C. Cells were then treated with urethane at various concentrations for 48 h at 37 °C. Each cell condition was tested in six replicate. During the final 4 h of the 48 h incubation, the supernatants were disposed of, and 100 μl of MTT (0.5 mg/ml) was added to each well. After 4 h, the MTT was disposed of, and 100 μl of DMSO was added to each well. Optical density (OD) was determined on a plate reader (BIO-IEK, Elx800) at 570 nm. Results of cell proliferation were presented as the percent of control cells.

2.5. Soft agar assay

Cell colony formation was analyzed by soft agar assay according to the previous procedures (Lee et al., 1990). Briefly, a bottom layer of 0.5% agarose (Promega, USA) containing 2 ml of DMEM medium was initially solidified in a 6-well culture plate at 4 °C. Next, 2 ml of 0.3% agarose solution containing 10,000 L929 or ρ° L929 cells plus 50 μg/ml uridine pretreated for 48 h with various concentrations of urethane were layered on top. Each cell condition was tested in triplicate. After incubation at 37 °C with 5% CO_2 atmosphere for 2 weeks, the colonies that contained over 30 cells were counted under a microscope.

2.6. Urethane-induced lung carcinogenesis model

The mice were administered freshly prepared urethane (ethyl carbamate; Sigma, St. Louis, MO) to induce lung carcinogenesis. In experimental design one, the mice received an intraperitoneal injection of urethane (800 mg/kg body weight) dissolved in sterile 0.9% NaCl. In experimental design two, the mice received intraperitoneal injections of urethane (800 mg/kg) twice weekly at intervals of 72 h for four weeks. Control mice received parallel intraperitoneal injections of saline. Animals were sacrificed at 2, 5, 10, 18, 26, 40, and 52 weeks after urethane exposure with age-matched controls, and their lungs were prepared for histological and mitochondrial dysfunction examination. Twenty mice in each group were examined at each time point. To investigate the role of mitochondrial dysfunction in carcinogenesis, animals were treated with 2,4-Dinitrophenol (DNP) (subcutaneous injection at 30 mg/kg) or genipin (intraperitoneal injection at 50 mg/kg) for eight weeks (weeks 2–9 of study) following the first dose of urethane.

2.7. Histopathological analysis

The animals were killed by intraperitoneal injection of 80 mg/kg pentobarbital. The lungs were immediately excised, and 10% buffered formalin was instilled to infiltrate and to fix the lung structures. The number of external nodules in each pulmonary lobe was counted after instillation of the fixative. After gross examination, a part of each lung lobe was preserved in 10% buffered formalin. Randomly selected, formalin-fixed lung tissues were routinely processed and embedded in paraffin. Histological sections (5 μm) were stained with hematoxylin and eosin and analyzed. Proliferative lesions in the lungs were classified as hyperplasia, adenoma or adenocarcinoma, based on recommendations published by the Mouse Models of Human Cancers Consortium (Nikitin et al., 2004).