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Original Articles

Tumorigenesis of smoking carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone is related to its ability to stimulate thromboxane synthase and enhance stemness of non-small cell lung cancer stem cells

Yi Liu [a,b,1], Shucai Yang [a,1], Ming-Yue Li [a], Runyue Huang [c], Calvin S.H. Ng [a], Innes Y.P. Wan [a], Xiang Long [d], Jun Wu [e], Bin Wu [e], Jing Du [d], Tony S.K. Mok [f], Malcolm J. Underwood [a,*], George G. Chen [a,*]

[a] Department of Surgery, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin N.T., Hong Kong
[b] Guangdong Key Laboratory for Research and Development of Natural Drugs, Guangdong Medical University, Zhanjiang, Guangdong, China
[c] The Second Affiliated Hospital, Guangzhou University of Chinese Medicine, Guangzhou, Guangdong, China
[d] Peking University Shenzhen Hospital, Shenzhen, Guangdong, China
[e] Department of Respiratory Medicine, Affiliated Hospital of Guang Dong Medical University, Zhanjiang, Guangdong, China
[f] Department of Clinical Oncology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin N.T., Hong Kong

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ABSTRACT

Lung cancer stem cells (LCSCs) play a critical role in lung cancer development, however, it is unknown whether thromboxane synthase (TXS) plays a role in the maintenance of LCSCs stemness. This study aimed to determine the *in vivo* role of TXS in lung cancer induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a smoking carcinogen. Results showed that ozagrel, a TXS blocker, suppressed NNK-induced lung tumors in mice. The expressions of CD133 and ALDH1A1 were positively associated with TXS. Similar results were observed in human NSCLC tumor samples. NNK significantly stimulated TXS and enhanced the generation of LCSCs, evident by the upregulation of CD133 and ALDH1A1 expression, and the increase in the number and size of tumor spheres. NNK also promoted the expression of LCSC-related molecules including β-catenin and Nanog. All these NNK-mediated effects could be offset by ozagrel. In the colony formation assay, NNK increased whereas ozagrel decreased the number of colonies. Collectively, LCSCs and TXS participate in NNK-induced lung cancer. Our data suggest that TXS is a promising therapeutic target as it is a key molecular in NNK-mediated stemness of LCSCs.

Introduction

More than 85% of lung cancers are non-small cell lung cancer (NSCLC) and the majority of lung cancers can be attributed to cigarette smoking. Among thousands chemicals generated from burning cigarette, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a nitro-samine, has been identified as the most abundant and potent carcinogen [1–5].

Thromboxane synthase (TXS), a key enzyme that converts prostaglandin H2 (PGH2) to thromboxane A2 (TXA2), is overexpressed in lung tumor tissues from smokers/ex-smokers with lung cancer [6,7]. Furthermore, *in vitro* TXS can activate cAMP response element binding protein through PI3K/Akt and ERK signaling pathways and subsequently contribute to the NNK-mediated survival and proliferation of lung cancer [7,8]. However, how TXS plays its role in NNK-induced lung cancer *in vivo* is unclear.

It has been reported that U46619, a stable TXA2 mimetic, can strongly stimulate differentiation, migration, and proliferation of mesenchymal stem cells (MSCs) [9,10] and the block of TXS prevents MSCs-induced resistance to chemotherapy [11]. On the other hand, NNK enhances breast cell carcinogenesis by enhancing mesenchymal and stem-like cell properties [12] and promotes sphere-formations in head and neck squamous cell carcinoma [13]. Therefore, we hypothesized that lung cancer stem cells (LCSCs) were involved in the tumorigenesis of NNK and that TXS was a crucial molecule in this progress.

In the present study, we firstly assessed whether TXS regulated the development of lung cancer induced by NNK *in vivo*. Then, we examined the levels of cancer stem cell (CSC) markers CD133 and aldehyde dehydrogenase 1 family member A1 (ALDH1A1) after NNK treatment in *in vivo* and *in vitro* models. An *in vitro* sphere

* Corresponding author. Tel.: 852 26323934; fax: 852 26450605.
E-mail address: gchen@cuhk.edu.hk (G.G. Chen).
[1] These authors contributed equally to this work.

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culture system for isolation and enrichment of non-small cell lung cancer stem cells (NSCLCSCs) was established to explore the underlying molecular mechanisms. We showed that the inhibition of TXS downregulated NSCLCSCs and suppressed lung tumor growth.

Materials and methods

Materials

NNK was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). The antibody against TXS, TXB2 enzyme immunoassay (EIA) kit and ozagrel were obtained from Cayman Chemical (Ann Arbor, MI). IWR-1 was purchased from Enzo Life Sciences (Farmingdale, NY). For other chemicals and reagents, please refer to Supplementary material.

Animal treatment

6-week-old A/J female mice (Bar Harbor, ME) were used in the study. 60 mice were randomly divided into six groups (Fig. 1). NNK was used to induce lung tumors as described previously [14]. Mice were treated with 3 different doses of ozagrel (15, 30 and 60 mg/kg/per day) via gavage-feeding five days per week for a total 8 weeks. The mouse experiments were approval by the animal research ethics committee of the Chinese University of Hong Kong and the guidelines for care and use of laboratory animals were strictly followed.

Cell lines and cell cultures

NSCLC NCI-H23 and NCI-H1299 cells were obtained from the American Type Culture Collection. The cells were cultured in DMEM (NCI-H23) or RPMI-1640 (NCI-H1299) supplemented with 10% inactivated FBS and incubated at 37 °C under 5% CO_2. In sphere culture system, NCI-H1299 cells were cultured in Cancer Stem Cell Media Premium (ProMab) to generate spheroid forming CSCs and the third-generation spheres were used for experiments. For details, please refer to Supplementary material.

TXB2 analysis

The levels of TXB2 in lung tissues and serum of A/J mice, and cell culture media were detected by an EIA kit from Cayman Chemical according to the manufacturer's instruction.

Biochemical test to assess the potential side effects of ozagrel

Mouse serum was collected and tested for aminotransferase (AST) activity, alanine aminotransferase (ALT) activity, creatinine (CRE) and blood urea nitrogen (BUN) using respective assay kits (Jiancheng Bioengineering, Nanjing, China).

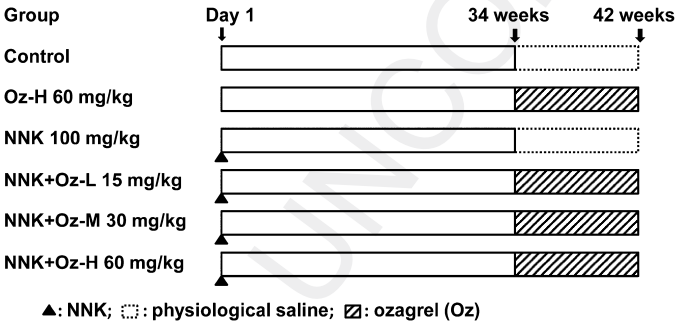

Fig. 1. 60 A/J mice were randomly divided into six groups. On day 1, the mice, except control and 60 mg/kg ozagrel (Oz-H) groups, were administrated with NNK (100 mg/kg of body weight) via intraperitoneal injection and the mice were maintained under standard conditions for 34 weeks. Then the Oz-H, NNK + Oz-L (15 mg/kg ozagrel), NNK + Oz-M (30 mg/kg ozagrel) and NNK + Oz-H groups were treated with ozagrel via gavage-feeding for five days whereas the control and NNK groups were given physiological saline by gavage. The treatment was stopped for two days before the next round of 5-day gavage-feeding of ozagrel was started. The treatment lasted for 8 weeks before the mice were sacrificed. The weight of mice was weighed weekly. The blood was drawn by direct venal puncture at the posterior vena cava. The lungs were dissected and the number of tumors in the lung surface was counted. The right lung was fixed and cut into 4 μm sections for hematoxylin and eosin (H&E) and immunohistochemistry (IHC) staining. The left lung was rapidly frozen in liquid nitrogen for others analysis.

Hematoxylin and eosin, and immunohistochemical staining

Hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining were performed as described previously [15].

Western blot analysis

Western blot was carried out according to previous publications [7,8].

Cell viability analysis

To investigate the effect of ozagrel on cell viability, 5×10^3 cells in 100 μl were seeded into a 96-well plate for over-night. The new medium containing 10 μM NNK or 10 μM NNK plus different concentrations of ozagrel (0.01, 0.1, 1, 10, 100, 1000 μM) was added into each well. After incubation for 24, 48, and 72 hours, cell viability was determined by MTT assay [7,8]. The percentage of living cells was defined as the ratio of the absorbance of the treated cells to the control cells and multiplied by 100 [(Absorbance of treated cells/Absorbance of untreated cells) × ××%].

Apoptosis assay

APO-DIRECTTM APOPTOSIS ASSAY kit was used for apoptosis assay by flow cytometer [7,8].

Sphere formation assay

The third-generation tumor spheres formed by NCI-H1299 were dissociated into single-cell suspension and plated in ultra-low adhesion 6 well plates at the density of 2500 cells/ml in Cancer Stem Cell Media Premium and allowed to grow for 10 days. Images of the spheres were taken using phase contrast microscope (Nikon) and total number and size of spheres more than 60 μm were counted. To study the effect of drugs on the self-renewal of NSCLCSCs, NNK and ozagrel were added to the respective wells once every 2 days.

Colony formation assay

Tumor spheres generated by NSCLCSCs were dissociated into single-cell suspension and plated in 6 well plates at a density of 500 cells per well in RPMI-1640 with 10% FBS. NNK and ozagrel were added to the respective wells once every 2 days. The plates were further incubated for 14 days at 37 °C with 5% CO_2 until colonies were visible. The colonies were stained with 0.01% crystal violet and counted under inverted microscopy.

Statistics analysis

SPSS19.0 was used for statistics analysis. Significance of differences in tumor incidence and tumor size were evaluated with Student's t test. Analyses of other data in different groups were determined by repeated measures ANOVA. $P < 0.05$ was considered as statistically significant.

Results

Block of TXS suppresses the development of NNK-induced lung cancer in A/J mice

To assess whether the development of NNK-induced lung tumor can be inhibited by ozagrel, a TXS inhibitor [16], NNK-treated mice were given ozagrel. The dose of ozagrel was based on the previous publications [16–18]. Both the incidence and the number of lung tumors on the lung surface were quantified at the end of the 42-week experiment. Compared with NNK treatment groups, the multiplicity in Control, NNK + Oz-M (ozagrel moderate dose) and NNK + Oz-H (ozagrel high dose) groups were very low (Fig. 2A, $P < 0.01$). Ozagrel treatment does-dependently reduced the number of tumors (Fig. 2A) but had no effects on the incidence (Fig. 2B). The results of H&E staining also showed that ozagrel treatment reduced the degree of malignant transformation in lung tissues (Supplementary Fig. S1).

To further confirm whether block of TXS would suppress the development of NNK-induced lung cancer, we examined the level of TXB2 in serum and lung tissues of A/J mice. TXB2 is a stable and biologically inactive metabolite of TXA2 which is extremely unstable in aqueous solution, being rapidly and non-enzymatically

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