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Tobacco specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone suppresses a newly identified anti-tumor IGFBP-3/IGFBP-3R system in lung cancer cells

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ABSTRACT

IGFBP-3 is a tumor suppressor whose expression is frequently suppressed in lung cancer. NNK, the most potent tobacco carcinogen, enhanced cell proliferation of BEAS-2B normal lung epithelial cells and concomitantly suppressed IGFBP-3 expression through DNA methylation. Decreased IGFBP-3 expression and elevated levels of phospho-Akt, phospho-p65-NF-κB, and cyclin D1 were detected in tobacco carcinogen-induced tumorigenic derivatives of BEAS-2B. Overexpression of IGFBP-3 in NNKA, one of the derivatives, suppressed NF-κB activity and induced apoptosis, which was hindered by knocking-down of endogenous IGFBP-3R, an IGFBP-3 specific receptor. These results suggest that NNK inhibits IGFBP-3 expression to abrogate anti-tumor actions of the IGFBP-3/IGFBP-3R system in smoking-induced lung cancer.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths in the world with more than 1 million deaths annually. The single most important risk factor for lung cancer development is smoking and it accounts for 85–90% of cases [1]. Among other carcinogens of cigarette smoking condensate (CSC), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is the most potent constituent. Subcutaneous injection of NNK induces tumors of the lungs in rodents [2–5]. When tracheal xenograft containing BEAS-2B cells, immortalized non-tumorigenic human bronchial epithelial cells, were exposed to NNK, the xenografts developed tumors with a multilayered epithelium that invaded the tracheal wall. Furthermore, the cell lines established from the tumors such as BEAS-2B-CSC 1170-I (CSC 1170-I) and BEAS-2B-NNK (NNKA), developed invasive adenocarcinoma in nude mice [6].

The molecular mechanisms of NNK-induced lung tumorigenesis have been studied in in vivo and in vitro systems. NNK has been demonstrated to cause transversion mutation in proto-oncogene K-ras in the lung tumors of mice [7,8] which has been shown to be associated with lung cancer among smokers [9,10]. Chromosomal alterations were more frequently observed in NNK-induced murine lung adenocarcinoma than in spontaneous lung tumors [11,12]. Those alterations have been detected in tobacco-related human lung adenocarcinoma [13,14]. NNK activates critical signaling pathways for cell proliferation and survival. NNK activates Akt to promote cell proliferation in NSCLC cell lines [15] and in normal human airway epithelial cells which results in a transformed phenotype [16]. NF-κB activation by NNK induced cell proliferation through upregulation of cyclin D1 in normal human bronchial epithelial cells [17]. NNK can also induce DNA methylation which may link to tumor suppressor gene silencing and cancer progression [18–20].

Insulin-like growth factor (IGF) binding protein-3 (IGFBP-3), the most abundant IGFBP in circulation, belongs to the IGF system [21] and is a potent p53-regulated tumor suppressor [21–24]. Recent epidemiological studies have demonstrated the relation between IGFBP-3 levels in circulation and risk of cancer. Lower IGFBP-3 levels in the circulation has been shown to be associated with a greater risk of common cancers such as lung, breast, prostate, and colorectal cancer [25–32], while inverse correlation of IGFBP-3 with cancer risk has been detected only after IGFBP-3 is adjusted to IGF-1 level [33]. Nonetheless, the involvement of IGFBP-3 in cell growth inhibition and induction of apoptosis in lung, breast, prostate, and colon cancer has been demonstrated in vivo and in vitro [34–41]. IGFBP-3 has been known to exert its anti-tumor effects IGF-dependently as well as independently. We have recently identified IGFBP-3R,

Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; IGF, insulin-like growth factor; IGFBP, IGF binding protein; NSCLC, non-small cell lung cancer; 5′-aza-dC, 5′-aza-2′-deoxycytidine; Ad, adenovirus; CSC, cigarette smoking condensate; m.o.i., multiplicity of infection.

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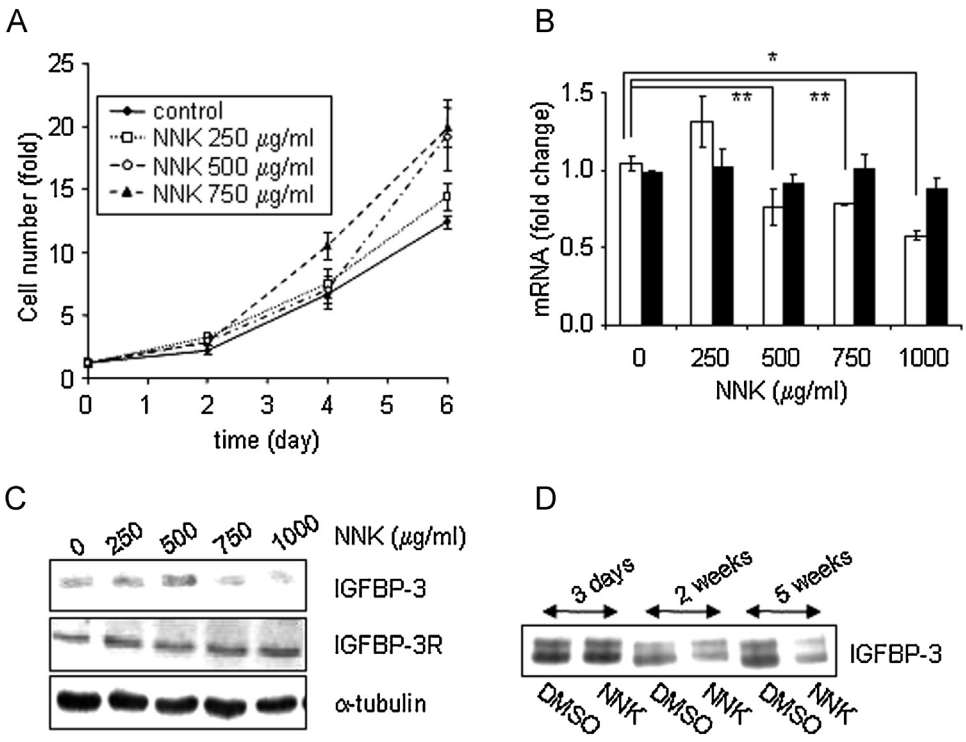

Fig. 1. NNK enhances BEAS-2B cell proliferation accompanying suppression of IGFBP-3 expression. (A) BEAS-2B cells were treated with NNK in the presence of 5% serum. The medium was changed every day with fresh NNK. DMSO was added to the control cells. The result is shown as fold increase compared with the cell number at day 0. Bars denote mean ± S.D. (B) BEAS-2B cells were treated with the indicated concentration of NNK in the absence of serum for 24 h. The mRNA levels of IGFBP-3 (open box) and IGFBP-3R (filled box) were measured by quantitative RT-PCR. GAPDH was used as an internal control. * and ** $P < 0.01$ and $P < 0.05$ vs. 0 μg/ml of NNK, respectively. Bars denote mean ± S.D. (C) BEAS-2B cells were treated with the indicated concentration of NNK in the absence of serum for 3 days. IGFBP-3 protein secretion in the conditioned media (CM) and IGFBP-3R expression in the cell lysates were analyzed by Western blot. (D) BEAS-2B cells were cultured in the absence of serum with/without 500 μg/ml of NNK for three different time periods. IGFBP-3 protein secretion in the CM was analyzed by Western blot. Equal amount of protein was loaded in each lane.

a novel cell death receptor for IGFBP-3, which mediates IGFBP-3's anti-tumor effects in breast and prostate cancers [38,39]. However, the specific mechanisms involved in suppression of the anti-tumor actions of IGFBP-3/IGFBP-3R system in tobacco carcinogen-induced lung cancer have yet to be elucidated.

We describe herein the impact of tobacco specific carcinogen on the IGFBP-3/IGFBP-3R system in lung cancer cells. We found that tobacco carcinogen NNK inhibits IGFBP-3 expression via hypermethylation, thereby abrogating anti-tumor actions of the IGFBP-3/IGFBP-3R system in lung cancer cells.

2. Materials and methods

2.1. Reagents

Anti-IGFBP-3 and anti-IGFBP-3R antibodies were generated and validated in our laboratory (Strategic BioSolutions, Newark, DE) [38,39]. Anti-phospho-Akt (S473), anti-Akt, anti-phospho-IκBα, anti-IκBα, anti-phospho-p65-NF-κB, anti-cyclin D1 and horseradish peroxidase-conjugated secondary antibodies were purchased from cell signaling. Anti- p65-NF-κB was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-tubulin was obtained from Sigma. NNK was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). 5′-Aza-2′-deoxycytidine (5′-aza-dC) and IKK-2 inhibitor IV were obtained from EMD Chemicals (Gibbstown, NJ).

2.2. Generation of recombinant adenovirus containing IGFBP-3 (Ad:IGFBP-3)

The AdEasy system (Quantum Biotechnologies, Quebec, Canada) was used to generate Ad:IGFBP-3, Ad:EV (empty vector), and Ad:LacZ as described previously [38]. Following amplification, lysates containing clonal recombinant Ad were prepared and purified by CsCl gradient centrifugation (Virginia Commonwealth University Virus Core Facility).

2.3. Cell culture

BEAS-2B was purchased from ATCC. BEAS-2B-CSC 1170-I (CSC 1170-I) and BEAS-2B-NNK (NNKA) were kindly provided by Dr. Phillip A. Dennis (National Cancer Institute). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech, Manassas, VA) supplemented with 10% FBS at 37 °C and 5% CO_2.

2.4. Transfection of shRNA

NNKA and CSC 1170-I cells were transfected with IGFBP-3R shRNA (pGIPZ-TMEM219shRNAmir) or control plasmid encoding non-targeting shRNA (Open Biosystems, Huntsville, AL) using Fugene 6 (Roche Applied Science, Indianapolis, IN). NNKA cells were further subjected to drug selection to obtain stable transfectants. The selection with 0.5 μg/ml of puromycin was started 24 h after the transfection.

2.5. Cell proliferation assay

BEAS-2B cells were daily treated with either NNK or DMSO in fresh DMEM containing 5% serum. NNKA cells were infected with either Ad:EV or Ad:IGFBP-3 in DMEM containing 5% FBS. The medium was changed every other day. Cell counting and WST-1 assay (Roche Applied Science) were performed at the indicated time.