Nicotine Does Not Enhance Tumorigenesis in Mutant K-Ras–Driven Mouse Models of Lung Cancer

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Abstract

Smoking is the leading cause of preventable cancer deaths in the United States. Nicotine replacement therapies (NRT) have been developed to aid in smoking cessation, which decreases lung cancer incidence. However, the safety of NRT is controversial because numerous preclinical studies have shown that nicotine enhances tumor cell growth in vitro and in vivo. We modeled NRT in mice to determine the effects of physiologic levels of nicotine on lung tumor formation, tumor growth, or metastasis. Nicotine administered in drinking water did not enhance lung tumorigenesis after treatment with the tobacco carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Tumors that develop in this model have mutations in K-ras, which is commonly observed in smoking-related, human lung adenocarcinomas. In a transgenic model of mutant K-ras–driven lung cancer, nicotine did not increase tumor number or size and did not affect overall survival. Likewise, in a syngeneic model using lung cancer cell lines derived from NNK-treated mice, oral nicotine did not enhance tumor growth or metastasis. These data show that nicotine does not enhance lung tumorigenesis when given to achieve levels comparable with those of NRT, suggesting that nicotine has a dose threshold, below which it has no appreciable effect. These studies are consistent with epidemiologic data showing that NRT does not enhance lung cancer risk in former smokers. Cancer Prev Res; 4(11); 1743–51. ©2011 AACR.

Introduction

Lung cancer is the leading cause of cancer related deaths in the United States (1). Smoking accounts for more than 90% of the lung cancer cases reported in men and 80% in women each year. K-Ras mutations are common in tobacco-related lung adenocarcinomas (2). K-Ras mutations enable Ras to remain in its active GTP-bound form, leading to activation of several signaling pathways including the phosphoinositide-3–kinase (PI3K), mitogen-activated protein kinase (MAPK), and Rac pathways (3). Patients with K-ras mutant lung cancer have a particularly poor prognosis (4), thus emphasizing the importance of smoking cessation to decrease lung cancer incidence and mortality (5).

The association of nicotine dependence with lung cancer is highlighted by the recent finding of genetic polymorphisms linked to both (6). Nicotine replacement therapy (NRT) is approved by the Food and Drug Administration to help smokers overcome nicotine dependence and is available in several forms such as gum, lozenges, and patches. When used at the recommended dosage and duration, NRT is thought to be a safer option than continued smoking (7, 8). However, preclinical models have shown that nicotine can activate components of the PI3K, MAPK, and Src pathways that increase lung cancer cell proliferation, survival, and migration (9–12). Activation of these pathways by nicotine can also partially transform epithelial cells, induce an epithelial–mesenchymal transition, and increase chemotherapeutic resistance (9, 13, 14). When extrapolated to former smokers, these data have raised the possibility that NRT might contribute to adverse clinical outcomes. However, many of these preclinical studies have used nicotine at higher doses than those achieved in heavy smokers.

The aim of this study was to assess tumor promotion by nicotine in murine models of mutant K-ras–induced lung tumors when nicotine was administered to mimic NRT. The following 3 models were used: a tobacco carcinogen–induced model, a transgenic model, and a syngeneic graft model. Nicotine was provided in the drinking water to achieve low steady-state concentrations. In each model, there was no evidence of tumor promotion by nicotine when administered to achieve levels comparable with NRT users.
These results suggest that NRT will not have an impact on the growth of human lung cancers bearing K-Ras mutations.

Materials and Methods

Mice

All animal studies were conducted under a protocol approved by the Animal Care and Use Committee of the National Cancer Institute. A/J and C57BL/6 (Charles River) mice were mated to generate F1 (AB6F1) mice that were used for all mouse experiments unless otherwise noted. For carcinogen studies, mice were divided into 4 groups, 10 mice per group. Beginning at 6 weeks of age, 2 groups received 3 weekly intraperitoneal injections of 100 mg/kg 4-[(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK; Toronto Research Chemicals). One week after the last NNK injection, one NNK group and one control group were given 100 μg/mL nicotine (N0267, Sigma-Aldrich) in their drinking water for 12 weeks. Water supplemented with nicotine was changed weekly and mice were sacrificed at 22 weeks of age.

Kras1LA2/+ mice (15) in a C57BL/6 background received nicotine in the drinking water or standard water starting at 3 weeks of age. Mice were sacrificed after a 2-week treatment. For longer chronic studies, mice began nicotine treatment at 6 weeks of age and were sacrificed at 12 weeks of age. For survival studies, mice began nicotine treatment at 6 weeks of age and were sacrificed when they exhibited signs of morbidity. At sacrifice, livers, lungs, kidneys, spleen, and obvious tumors were removed and preserved in neutral-buffered formalin (NBF). For all studies, lungs were inflated using 10% NBF and peripheral lung tumors were fixed in NBF for histology. Lungs were embedded in paraffin and used for all mouse experiments unless otherwise noted. For survival studies, mice were mated to generate F1 (AB6F1) mice that were used for all mouse experiments unless otherwise noted. For carcinogen studies, mice were divided into 4 groups, 10 mice per group. Beginning at 6 weeks of age, 2 groups received 3 weekly intraperitoneal injections of 100 mg/kg 4-[(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK; Toronto Research Chemicals). One week after the last NNK injection, one NNK group and one control group were given 100 μg/mL nicotine (N0267, Sigma-Aldrich) in their drinking water for 12 weeks. Water supplemented with nicotine was changed weekly and mice were sacrificed at 22 weeks of age.

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For syngeneic allograft studies, AB6F1 mice between 6 and 10 weeks of age were injected subcutaneously in the flank with 1 × 105 CL13, IO33, or CL25 cells. One day after injection, mice were randomized to receive nicotine at 100 μg/mL in their drinking water or control water starting at 3 weeks of age. Mice were sacrificed after a 2-week treatment. For longer chronic studies, mice began nicotine treatment at 6 weeks of age and were sacrificed at 12 weeks of age. For survival studies, mice began nicotine treatment at 6 weeks of age and were sacrificed when they exhibited signs of morbidity. At sacrifice, livers, lungs, kidneys, spleen, and obvious tumors were removed and preserved in neutral-buffered formalin (NBF). For all studies, lungs were inflated using 10% NBF and peripheral lung tumors were counted and measured as previously described (16).

Immunoblotting and immunohistochemistry

Cell lysates were made in 4% SDS, 125 mmol/L Tris, pH 6.8, 20% glycerol, and briefly sonicated. For tissue lysates, liver, lungs, or tumors were flash frozen and then ground in RIPA buffer (Sigma) with added protease and phosphatase inhibitors. Immunoblotting was conducted as previously described (19). Primary antibodies Akt (9272), pS473-Akt (4060), pT202/Y204-Erk1/2 (4376), pS235/236-ribosomal protein S6 (2211), and α-tubulin (2125) were obtained from Cell signaling Technology and used according to manufacturer’s protocol. The antibody directed at Foxp3 was obtained from eBioscience (14-5773-82). Secondary antibody (Vectastain) and DAB detection (Sigma) were used as previously described (20).

Survivin staining was quantified by counting the number of survivin-positive cells in four 400× high-power fields (HPF) per tumor. Numbers were averaged for all mice with at least 5 mice per group.

Results

Levels of nicotine comparable with NRT do not enhance tobacco carcinogen–induced lung tumorigenesis in mice

The A/J mouse has been widely used to study lung tumorigenesis and is susceptible to NNK-induced lung tumors. However, A/J mice are reported to show little...
preference for nicotine in drinking water, whereas C57BL/6 mice show a high preference (22). To generate mice that are both susceptible to NNK-induced lung tumors and have a preference for nicotine, A/J and C57BL/6 mice were crossed to generate AB6F1 mice. These mice were treated with NNK followed by nicotine administration in the drinking water to simulate a former smoker on NRT. Nicotine (100 μg/mL) was chosen because concentrations above this did not result in increased daily nicotine dose to C57BL/6 mice (22). Nicotine treatment did not alter tumor multiplicity or size as assessed by peripheral tumor number and tumor burden (Fig. 1). As expected, tumor multiplicity was lower in these AB6F1 mice than in pure A/J mice that develop approximately 25 lung tumors per mouse and increased compared with pure C57BL/6 that do not develop lung tumors after NNK (23, 24). Nicotine had no effect on tumor incidence, even though 1 of 10 mice in the nicotine-alone group developed one lung tumor. When this group was expanded to 30 mice, no additional mice developed lung tumors. Nicotine did appear to increase tumor incidence in NNK-treated mice but the difference was not statistically significant (Fig. 1). These studies were repeated after decreasing the dose of NNK to a single 100 mg/kg injection to highlight any effect of nicotine on incidence. The single dose of NNK led to 37% incidence of lung tumors. A single dose of NNK followed by nicotine at the same dosing schedule above led to a 31% incidence, which was not statistically different from the single dose NNK alone group (the Student t test; P = 0.78, n = 13–19 per group; data not shown).

Serum cotinine levels measured at sacrifice averaged 137 ng/mL, which is comparable with 22 mg nicotine patch users (25). Females metabolize nicotine faster than males due to the influence of estrogen on CYP2A6 (26). When cotinine levels were stratified on the basis of gender, females achieved an average steady-state level of 100 ng/mL whereas the males averaged 200 ng/mL (Supplementary Fig. S1). This concentration range of nicotine did not have observed toxicities.

Nicotine can activate the MEK/ERK and PI3K/PROTEASE pathways in vitro (9, 27). In vivo, nicotine increased P5473-Akt expression in lung tumors, but this did not correlate with increased Ki-67 or pT308-Akt staining (Fig. 2A and Supplementary Fig. S2). Because increased phosphorylation of Akt at both sites is necessary for full Akt kinase activation and increased proliferation, phosphorylation of only S473 is likely insufficient to promote tumor growth. No modulation of p-S6 or p-Erk was observed. K-Ras–mediated lung tumorigenesis requires the presence of Foxp3+/CD3+ regulatory T cells in the tumor microenvironment (28). Although nicotine alone has been reported to increase the suppressive effects of regulatory T cells (29), nicotine did not alter Foxp3+/CD3+ cell number in lung tumors or surrounding normal lung tissue (Supplementary Fig. S2)

Levels of nicotine comparable with NRT do not enhance mutant K-Ras–mediated lung tumorigenesis

NNK-induced lung tumors in A/J mice exhibit K-ras mutations but are primarily adenomas that only progress to adenocarcinomas at later ages (30). A genetically engineered mouse model of mutant K-Ras (KrasG12D) was used to assess the effect of nicotine on tumor growth and progression. In this model, tumors are apparent as early as 2 weeks of age and progress to adenocarcinomas within several months, coincident with greatly increased tumor size (31). Two weeks of nicotine starting at 3 weeks of age did not change tumor multiplicity or tumor burden (Fig. 3A and data not shown). To determine whether nicotine could increase tumor progression in older mice, 6-week-old mice were treated with nicotine for 6 weeks. There was no difference in peripheral tumor multiplicity, tumor size, or tumor burden in the presence of nicotine, although tumor number and size were increased relative to younger mice (Fig. 3A). When initiated at 6 weeks of age and continued until death, nicotine also did not alter the overall life span of these mice (Fig. 3B). Cotinine levels were comparable with the AB6F1 mouse model (data not shown). In lung tumors from these mice, nicotine did not alter the activation of proteins associated with survival such as S6, Akt, and extracellular signal-regulated kinase (Erk) and did not increase Ki-67 staining (Fig. 3C).
mice. Three NNK-derived cell lines were used that exhibited differences in endogenous expression of survival pathways (Fig. 4A). IO33 and CL13 cells show relatively high expression of phosphorylated Akt compared with CL25. CL13 cells also have a mesenchymal phenotype as shown by a lack of E-cadherin expression and spindle shape in culture (Fig. 4A and data not shown). Oral nicotine did not enhance tumor growth of any of the 3 cell lines in AB6F1 mice (Fig. 4B). These same cell lines were subjected to an intraperitoneal nicotine dosing regimen that had previously been shown to enhance xenograft tumor growth (10). Intraperitoneal dosing of nicotine did not enhance tumor growth of IO33 or CL25 cells (Fig. 5). Approximately 10% of AB6F1 mice died within 24 hours of 1 mg/kg intraperitoneal nicotine injection (data not shown); therefore, subsequent experiments were done with lower doses. Intraperitoneal injections of nicotine at 0.6 to 1.0 mg/kg caused severe acute reactions in all mice that included tachypnea and immobility. Acute reactions to intraperitoneal nicotine lasted 10 to 15 minutes, after which time, the mice appeared to recover (data not shown). In addition to forming primary tumors, subcutaneously injected CL25 and CL13 cells can metastasize to the lung. Similar to its lack of effect on primary tumors, nicotine did not substantially increase the incidence of metastasis in either cell line (Fig. 4C). The apparent decrease in metastasis of nicotine-treated CL13 cells was not statistically significant. Xenograft tumors from nicotine-treated mice did not have activated Akt or Erk survival pathways (Fig. 4D and Supplementary Fig. S3).

Nicotine does not promote growth of NNK-induced mouse lung tumor cell lines in vitro

Because nicotine can rapidly stimulate survival pathways in vitro, the NNK-derived cell lines were treated with varying...
doses of nicotine to determine whether they might be refractory to these stimulatory effects of nicotine. Nicotine activated the Akt and Erk pathways at low physiologic nicotine doses in CL25 cells when they were serum deprived (Fig. 6A). However, activation was not observed when nicotine was added to cells grown in 5% FBS (Fig. 6B). The dependence of serum was confirmed when proliferation was analyzed with or without serum starvation. Nicotine did not promote cellular proliferation in low serum (data not shown), and there was no effect when nicotine was added in the presence of 5% FBS (Fig. 6D). These results show that physiologically relevant concentrations of nicotine do not promote cell proliferation \textit{in vitro} under normal growth conditions (5% FBS).

**Discussion**

We used 3 mouse models to show that oral nicotine, at steady-state levels relevant to NRT, does not enhance lung tumorigenesis after NNK exposure and does not enhance growth or metastasis of nascent lung tumors bearing K-Ras mutations. The NNK-induced lung tumor model is particularly relevant because it models a former smoker using NRT. The main clinical implication of these studies is that doses of nicotine that mirror use of NRT may not be harmful in the setting of prior tobacco use, but they shed no insight into possible effects of NRT in current smokers. Similar results have been reported by Murphy and colleagues, who show that oral administration of low doses of nicotine has no effect on NNK-induced lung tumorigenesis (32). These investigators varied the timing of nicotine in relation to NNK exposure as well as length of treatment and observed no differences in tumor multiplicity, size, or histology. Our study and that of Murphy and colleagues are in conflict with prior studies that showed that nicotine augmented NNK-induced lung tumors and/or lung tumor xenograft growth and metastasis (10). We believe the principal difference in the outcomes of these studies is that studies that show a...
tumor-promoting effect of nicotine used high intraperitoneal dosing that may not be reflective of sustained, lower nicotine doses typical of NRT.

For NNK, there is a threshold effect for lung carcinogenesis in mice, where doses of 2 µmol or below do not have appreciable tumorigenic activity, whereas doses above 2 µmol show a linear correlation with lung tumor multiplicity (33). A similar phenomenon may occur for nicotine where low doses or low dose rates do not have appreciable tumor promotion activity whereas larger doses do. In support of this, one study found no effect on growth of 3 small-cell lung tumor xenografts using Alzet minipumps to deliver 20 or 200 µg of nicotine per day (34). In contrast, high-dose intraperitoneal nicotine 3 times per week for 28 weeks in NNK-treated mice increased both lung tumor number and tumor size (10). In the studies presented here, NRT-level nicotine did not augment either lung tumor number or size following NNK exposure. The previous study used intraperitoneal dosing of nicotine, which delivered approximately 8 times the daily dose used here in a single bolus dose; this study also used 40% higher NNK dosing and more than twice the duration of nicotine treatment. In an attempt to replicate those studies and compare intraperitoneal dosing schedule with oral dosing, all injected mice developed severe acute respiratory distress and/or seizures and several mice died within 24 hours (data not shown). Lower doses of intraperitoneal nicotine did not enhance syngeneic tumor growth, similar to what was observed for oral dosing.

Figure 4. Nicotine does not increase tumor growth in allograft models. A, immunoblot analysis of NNK-induced mouse lung tumor cell lines in the absence of nicotine. B, tumor volume of IO33, CL13, and CL25 allograft tumors in AB6F1 mice with or without nicotine (Nic) administration (n = 5). C, incidence of lung metastasis at completion of the allograft study. The percentage of mice with metastatic tumors were identified and quantified from hematoxylin and eosin slides. D, immunoblot analysis of components of the PI3K and MAPK pathways in CL25 allograft tumors. Tumors were harvested at sacrifice upon completion of the study. Con, control.

Figure 5. Intraperitoneal injection of nicotine does not increase subcutaneous tumor growth. (A) IO33 and (B) CL25 allograft tumor growth curves after intraperitoneal injection of nicotine (IP) or water (control) or oral nicotine (PO). Any differences are not significant.
In vitro, nicotine has been shown to stimulate growth-promoting pathways that are frequently activated in lung tumors, particularly the mutant K-Ras–associated PI3K/AKT and MEK/ERK pathways (9, 27, 35). In normal lung and lung tumors, nicotine can bind to nicotinic acid receptors potentially stimulating these growth-promoting pathways (9, 36). Nicotine increased Akt, Erk, and mTOR pathway activation in vitro, but these effects were largely dependent on the absence of serum (Fig. 6), where basal levels of pathway activation are lower. In NNK-treated mice, nicotine partially enhanced Akt activation but this was not sufficient to propagate to the mTOR pathway (p-S6 in Fig. 2). In addition, these pathways were not elevated in tumors from the syngeneic model (Fig. 4D and Supplementary Fig. S3). This suggests that activation of these pathways by nicotine in vitro in low serum may not be relevant to what occurs with physiologic levels of nicotine in vivo.

Another consideration for these types of studies is strain background. Previous studies have shown stimulation of tumor growth in BALB/c, C57BL/6 and nude mice using intraperitoneal dosing of nicotine, or in BALB/c using a nicotine patch (10, 37, 38). Recessive traits relating to nicotine metabolism or response may be present in inbred strains. Here, AB6F1 mice were used in most studies. Because of the mixed background, there may be fewer strain-dependent variables than when using pure backgrounds. Likewise, there may be certain

**Figure 6.** Nicotine only activates components of PI3K and MAPK under serum starvation conditions. Immunoblotting analysis of C125 cells after 1-hour nicotine treatment at indicated doses in 0.5% serum (A) or 5% serum (B). C, SRB assay of C125 cells in 5% serum. Prior to nicotine treatment, OD570 values were approximately 0.1. The first bar for each time point is untreated cells. Nicotine doses (from left to right for each time point) were 1, 10, 100 nmol/L, 1, and 10 μmol/L. Any differences are not significant. DMSO, dimethyl sulfoxide.
populations that are at higher risk for nicotine-stimulated tumor growth as there are for risk for nicotine dependence. However, epidemiologic studies have indicated that large populations are not at increased risk of lung cancer because of NRT (8). The studies presented here support those findings using multiple mouse models and physiologic administration of nicotine.

References


Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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