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Cancer Prevention Research

Research Article

See Perspective on p. 1719 and 1724

Chronic Nicotine Consumption Does Not Influence 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone–Induced Lung Tumorigenesis

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Abstract

Nicotine replacement therapy is often used to maintain smoking cessation. However, concerns exist about the safety of long-term nicotine replacement therapy use in ex-smokers and its concurrent use in smokers. In this study, we determined the effect of nicotine administration on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumors in A/J mice. Female mice were administered a single dose of NNK (10 μmol) and 0.44 μmol/mL nicotine in the drinking water. Nicotine was administered 2 weeks prior to NNK, 44 weeks after NNK, throughout the experiment, or without NNK treatment. The average weekly consumption of nicotine-containing water was 15 ± 3 mL per mouse, resulting in an estimated daily nicotine dose of 0.9 μmol (0.15 mg) per mouse. Nicotine administration alone for 46 weeks did not increase lung tumor multiplicity (0.32 ± 0.1 vs. 0.53 ± 0.1 tumors per mouse). Lung tumor multiplicity in NNK-treated mice was 18.4 ± 4.5 and was not different for mice consuming nicotine before or after NNK administration, 21.9 ± 5.3 and 20.0 ± 5.4 tumors per mouse, respectively. Lung tumor multiplicity in animals consuming nicotine both before and after NNK administration was 20.4 ± 5.4. Tumor size and progression of adenomas to carcinomas was also not affected by nicotine consumption. In addition, nicotine consumption had no effect on the level of O^6-methylguanine in the lung of NNK-treated mice. These negative findings in a commonly used model of human lung carcinogenesis should lead us to question the interpretation of the many *in vitro* studies that find that nicotine stimulates cancer cell growth. *Cancer Prev Res; 4(11); 1752–60. ©2011 AACR.*

Introduction

Tobacco use results in more than 400,000 deaths annually in the United States; 157,000 of these are because of lung cancer (1, 2). The use of nicotine replacement therapy (NRT) to aid smoking cessation efforts would significantly decrease the number of tobacco-related deaths (3). However, concerns exist about the safety of long-term nicotine use in ex-smokers as well as its concurrent use with smoking. It has been suggested that nicotine is a driver of cancer growth, the "estrogen of lung cancer" (4). Nicotine is a ligand for the nicotinic acetylcholine receptor (nAChR) and has been shown to increase cell proliferation, inhibit apoptosis and enhance angiogenesis, and stimulate cancer cell growth (5–7). Therefore, the effect of nicotine on tobacco-induced lung cancer is of concern to the public health community.

In animals, nicotine alone is not a carcinogen (8). A two-year study of rats chronically exposed to inhaled nicotine did not result in any increased frequency of tumors in any tissue (9). A small number of lung tumors were induced in hamsters exposed to nicotine in the presence of hypoxia (10). More recently, nicotine has been reported to promote tumor growth and metastasis in cancer xenograft models (5, 11–13). However, animal cocarcinogenesis studies with nicotine have produced mixed results, some studies show evidence for cocarcinogenicity and others have shown no effect or a protective effect of nicotine (8, 12).

The effects of nicotine on cell proliferation, apoptosis, and angiogenesis are mediated by the interaction of nicotine with the nAChR (6, 7) and genome-wide association studies have implicated a gene locus (*15q25*) that encodes 3 nAChR subunits in lung cancer risk (14–16). Some investigators have suggested that this association is a direct link to lung cancer, independent from any effect on nicotine

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addiction and smoking intensity (7, 17). The possible direct link between nAChR and lung cancer has heightened the concern of U.S. Food and Drug Administration and public health officials in promoting long-term use of NRT (18). Nevertheless, a direct link has not been established. A meta-analysis in nonsmokers shows no association of the 15q25 locus with lung cancer, and data from the Lung Health Study do not support a link between NRT and lung cancer (19, 20). Also of note is a recent study in which chronic nicotine exposure, in contrast to short-term exposure, impairs angiogenesis and downregulates the nAChR (21).

In contrast to the above concerns, nicotine has been suggested to protect smokers from tobacco-induced carcinogenesis (22–24). Specifically, it has been hypothesized that nicotine competitively inhibits enzymes that catalyze the activation of the tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), as these enzymes also catalyze the metabolism of nicotine. The primary pathway of nicotine metabolism is cytochrome P450 2A6-catalyzed oxidation to the nicotine $\Delta^{5'(1')}$ iminium ion and further oxidation to cotinine and *trans*-3′-hydroxycotinine (25–27). P450 2A6 is also a catalyst of NNK α-hydroxylation (28). α-Hydroxylation generates reactive intermediates that alkylate DNA to adducts that result in mutagenic bases, initiating the carcinogenic process (8). P450 2A6 is a poor catalyst of NNK α-hydroxylation; however, the closely related extrahepatic enzyme, P450 2A13 is an excellent catalyst (28). P450 2A13, also an efficient catalyst of nicotine oxidation (26), is expressed in the human lung and likely contributes to the metabolic activation of NNK in smokers (29). Nicotine is not only a competitive inhibitor of P450 2A6 and P450 2A13 catalyzed reactions, it also irreversibly inactivates both enzymes during its metabolism (27). Therefore, nicotine metabolism in the lung may lead to a decrease in P450 2A13–catalyzed activation of NNK, decreasing DNA adduct formation and potentially protecting smokers from NNK carcinogenesis.

The A/J mouse is an excellent model to study modifiers of NNK-induced lung carcinogenesis (30, 31). In the mouse, nicotine and NNK are metabolized by the P450 2A6/2A13 ortholog, P450 2A5 (26). P450 2A5 present in both the lung and the liver catalyzes the bioactivation of NNK. In the mouse, the pathway critical to NNK-induced tumorigenesis is α-hydroxylation of the methylene carbon adjacent to the nitroso group (32, 33). This pathway leads to DNA methylation, and the persistence of O^6-methylguanine (O^6mG) adducts results in lung tumorigenesis. There is a strong correlation between O^6mG levels in the lung and the multiplicity of lung tumors in the NNK-treated A/J mouse (33). Also, when a lung-specific P450 reductase knockout mouse with no P450 activity in the lung is treated with NNK, both the number of lung tumors per mouse and the level of O^6mG DNA adducts in the lung are significantly reduced relative to NNK-treated wild-type animals (34). An inhibitor of P450 2A5 activity in the lung would be expected to have similar effects.

The study presented here was designed to test the following hypotheses: (i) that sustained exposure to nicotine prevents or delays tumor initiation in A/J mice treated with NNK, and (ii) that when tumor initiation occurs in this model, nicotine accelerates tumor growth by reducing apoptosis, promoting angiogenesis, and altering the tumor microenvironment. The effect of chronic nicotine administered in the drinking water on P450 2A5 activity and NNK-induced lung tumorigenesis was determined.

Materials and Methods

Chemicals, reagents, and diet

(*S*)-Nicotine hydrogen tartrate, NNK, coumarin, and other reagents were purchased from Sigma Aldrich. O^6mG was a gift from Stephen Hecht (Masonic Cancer Center, University of Minnesota). Mouse diets (AIN-93G and AIN-93M) were purchased from Harlan Teklad.

Animal studies

Four-week-old female A/J mice were obtained from the Jackson Laboratories. Mice were housed under specific pathogen-free conditions and allowed to acclimate for 7 days before experimental procedures. Experiments were carried out with approval from the University of Minnesota Institutional Animal Use and Care Committee. Husbandry practices and diet followed protocols described previously (35). Two experiments were carried out. The first was a 2-week study to determine the consumption of nicotine in the drinking water with and without saccharin. Saccharin has been used in previous studies to mask the taste of nicotine. Experiment 2 was carried out in 2 parts to determine the effect of chronic nicotine on NNK metabolism and DNA adduct formation and on NNK-induced tumorigenesis.

Experiment 1. Female A/J mice, 5 weeks of age, were randomized into 6 groups of 5 mice each. Treated groups received nicotine hydrogen tartrate (100 or 200 μg/mL) in the drinking water with or without 2% saccharin as described (5). Fresh water with the appropriate concentration of nicotine and/or saccharin was provided twice weekly for 2 weeks, and water consumption for each cage was measured at the same intervals. Sodium potassium tartrate was used as a control for animals that did not receive nicotine. Nicotine disposal was in accordance with state and local regulations. Urine was collected daily by gently massaging the abdomen to stimulate urination. Urine was not obtained from every mouse each day. Mice were euthanized according to the guidelines of the American Veterinary Medical Association after 2 weeks and blood collected immediately postmortem by cardiac venipuncture. Plasma levels of cotinine were determined for each mouse, and urine pooled by group was analyzed for cotinine.

Experiment 2. Groups of 40 mice (5 weeks of age, 5 mice per cage) were assigned to 1 of 6 experimental conditions as illustrated in Fig. 1. Nicotine hydrogen tartrate [200 μg (0.44 μmol)/mL] and sodium potassium tartrate were provided in drinking water as described above. Water consumption per cage per week was measured, and consumption per mouse was estimated on the basis of the number of mice per cage. After 2 weeks, each mouse in groups 3 to 6

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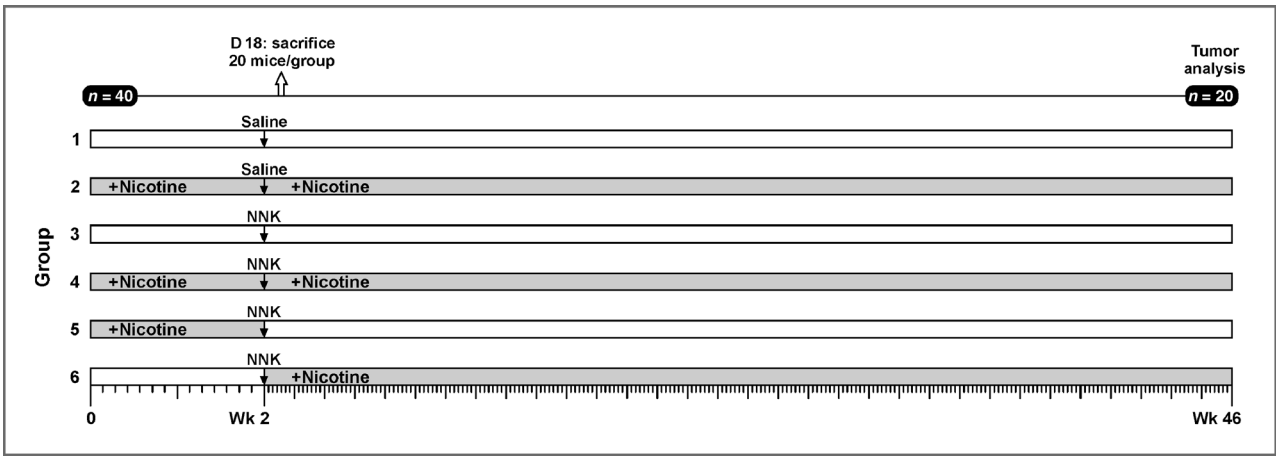

Figure 1. Experimental protocol to assess the effect of chronic nicotine consumption on the initiation and progression of NNK-induced lung tumorigenesis in the A/J mouse. Female mice were provided water containing nicotine (0.44 μmol/mL) as their sole source of drinking water throughout the study (groups 2 and 4), prior to NNK administration (group 5) or after NNK administration (group 6). Groups 1 and 3 did not receive nicotine and group 5 did not receive nicotine after week 2. Four days following NNK or saline treatment, 20 of the 40 mice in each group were euthanized for the determination of DNA adduct levels and P450 2A5 activity.

was administered with a dose of 2 mg (10 μmol) NNK in 0.1 mL of PBS as a single intraperitoneal injection (36). Mice that were not injected with NNK (groups 1 and 2) received an intraperitoneal injection of 0.1 mL PBS solution. Immediately following the administration of NNK or saline, nicotine-containing drinking water was provided to animals in group 6 and was replaced with non-nicotine–containing water for animals in group 5. Groups 2, 4, and 6 were provided nicotine-containing water for the remainder of the experiment. Blood and urine were collected as above for the first 2 weeks. Subsequently, blood and urine were collected from each animal in the nicotine consuming groups at least once a month. Twenty mice from each group were euthanized 96 hours after the NNK injection. Blood was collected immediately postmortem by cardiac venipuncture, and liver and lung tissues were harvested and frozen on dry ice and stored at −80°C until analysis for P450 2A5–catalyzed metabolism and O^6mG levels. The remaining mice were maintained for 44 weeks, at which time they were euthanized. Blood was collected immediately postmortem by cardiac venipuncture.

Tumor multiplicity and classification

Tumor multiplicity was determined grossly as described (35). Briefly, immediately upon sacrifice, lungs were rinsed with cold PBS solution, excised *en bloc*, and tumors counted using a dissecting microscope. Tumors were scored on the basis of location (lung lobe) and the widest tumor diameter (<0.5, 0.5–1.0, 1.1–2.0, and >2 mm). The entire pulmonary tree from 5 animals and a hemisection from 5 animals in groups 1 and 2 were flash frozen in liquid nitrogen and stored at −80°C for further enzyme and tumor analysis. The remaining sections were fixed by immersion in 10% neutral-buffered formalin for 24 hours and subsequently transferred to 70% ethanol for long-term preservation. For histopathology evaluation, lung sections were embedded in paraffin blocks. Three step sections, each 150 μm apart, were obtained from the blocks and stained with hematoxylin and eosin for microscopic analysis. Tumors were categorized according to criteria of the Mouse Models of Human Cancers Consortium (37). Briefly, alveolar hyperplasia was diagnosed by an increase in the number of cuboidal or columnar cells in an alveolus without cellular atypia. Adenomas were well circumscribed, less than 5 mm in diameter areas of proliferative cuboidal to columnar cells lining an alveolus. Adenomas with dysplasia (preinvasive lesions) showed marked cellular atypia and/or squamous metaplasia. Lesions that were larger than 5 mm in diameter and showed invasion into adjacent airways, blood vessels, or alveoli were classified as carcinomas.

Statistics

Descriptive statistics were used to enumerate water consumption, tumor multiplicity, and progression (total tumor number, size distribution, and pathologic classification). Data were analyzed using the 2-tailed Student *t* test and one-way ANOVA. To account for variance in lung lobes examined because of fragmentation and loss during processing, data were normalized per lung lobe. Significance was set at $P < 0.05$. Poisson regression models also were used to examine the effect of group on tumor multiplicity, the distribution of tumor types, and tumor diameters, after adjusting for plasma levels of nicotine metabolites.

Lung and liver P450 2A5 metabolism and lung O^6mG levels

Lung and liver microsomes were prepared as described previously (38). Half the lung or liver was used, and the microsomal pellet was resuspended in a final volume of 0.2 mL. Coumarin 7-hydroxylation activity, used to assess P450 2A5 activity, was quantified as previously described (39). Microsomes (5–20 μL) were incubated with 20 μmol/L coumarin and a NADPH generating system (0.4 mmol/L NADP, 10 mmol/L glucose-6-phosphate, 0.4 units/mL glucose phosphate dehydrogenase) in 50 mmol/L Tris, pH 7.4,

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for 10 to 20 minutes at 37°C. The reaction was stopped with 20 μL 15% trichloroacetic acid. DNA was isolated from half a lung (60–100 mg tissue) using the Puregene DNA Purification Kit (Qiagen. O^6mG levels were determined as previously described (40). The urine (1–5 μL) and plasma (25 μL) were analyzed for nicotine, cotinine, and *trans*-3′-hydroxycotinine by liquid chromatography/tandem mass spectrometry (LC/MS-MS) as previously described (41).

Results

Consumption of nicotine in the drinking water

In a 2-week pilot study, mice were provided nicotine in the drinking water with or without saccharin (group 1A/B, controls; group 2A, 0.22 μmol/mL nicotine; group 3A, 0.44 μmol/mL nicotine; group 2B, 0.22 μmol/mL nicotine with 2% saccharin; and group 3B, 0.44 μg/mL nicotine with 2% saccharin). The urinary concentrations of cotinine varied widely across the days of collection. Cotinine values ranged from 80 to 3,820 ng/mL and appeared to be independent of both saccharin supplementation and nicotine concentration. Plasma cotinine concentrations ranged from 3 to 45 ng/mL and were higher in animals consuming saccharin (group 2, 7 ± 4 ng/mL; group 3, 12 ± 8 ng/mL; group 2B, 19 ± 18 ng/mL; group 3B 22 ± 13 ng/mL). However, saccharin did not seem to significantly influence average water consumption, which ranged from 1.6 to 2.2 mL/mouse/d. The differences observed in cotinine plasma concentrations are likely because of differences in the time of nicotine consumption. Therefore, saccharin was not used in the tumorigenesis experiment.

For the first 4 weeks of the tumorigenesis study, there was no difference in water intake between nicotine consuming and non-nicotine consuming groups (Fig. 2A–C). However, water consumption was significantly decreased in the groups consuming nicotine over the next 42 weeks. The difference was less pronounced in the NNK-treated animals than in saline controls (Fig. 2A vs. B or C). None of the animals displayed clinical signs of dehydration. The average weekly water consumption per mouse over weeks 2 to 46 in the nicotine consuming animals was 15 ± 3 mL per mouse (Fig. 2). Therefore, the estimated daily dose of nicotine per mouse was 0.9 μmol (0.15 mg) per day, a significantly higher dose per kg than the approximately 20 mg/d consumed by a 70-kg, one-pack-a-day smoker.

Urinary nicotine, cotinine, and *trans*-3′-hydroxycotinine concentrations for weeks 1 and 2 and month 5 of nicotine consumption, presented in Table 1, are consistent with this high dose of nicotine. There was no difference in urinary nicotine, cotinine, or *trans*-3′-hydroxycotinine concentrations among the groups, and the mean values for months 2 through 10 were not significantly different than those at 5 months (data not shown). As in the 2-week study, urinary cotinine concentrations varied greatly across animals. The variation was somewhat less after several weeks of consumption. The urinary concentrations of nicotine were as much as 6-fold lower than cotinine and were also quite variable, ranging from 22 to 10,500 ng/mL. This is likely because of the time of urine collection relative to nicotine consumption and the relatively fast metabolism of nicotine in the mouse. The concentration of the primary cotinine metabolite *trans*-3′-hydroxycotinine was 4 to 10 times greater than cotinine.

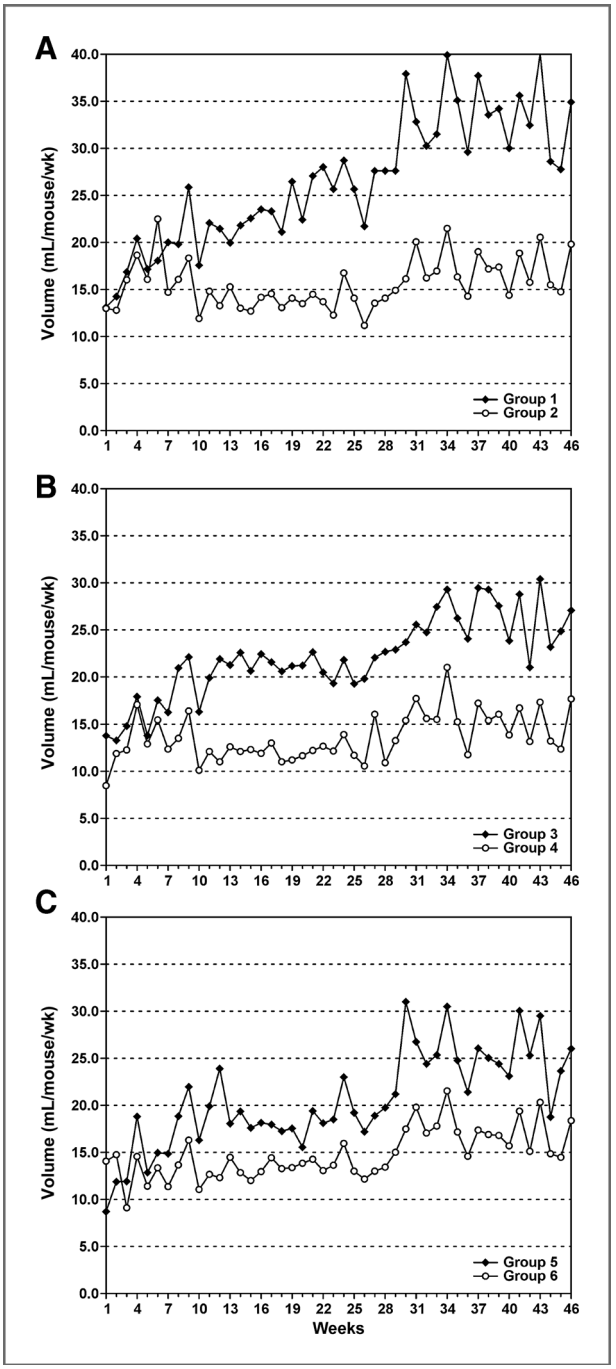

Figure 2. A–C, average weekly water consumption per mouse by group (described in Fig. 1). Water consumption for each cage was measured twice per week.

The plasma concentrations of nicotine, cotinine, and *trans*-3′-hydroxycotinine were determined at the 2

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Table 1. Concentration of nicotine, cotinine, and *trans*-3′-hydroxycotinine in the urine of nicotine consuming mice[a]

Group	Treatment[b]	Time	Nicotine, ng/mL		Cotinine, ng/mL		*Trans*-3′-hydroxycotinine, ng/mL	
			Mean	Range	Mean	Range	Mean	Range
2	Nicotine (0–46 wks)/saline	1–2 wks[c] ($n = 26$)	1,360 ± 1,040	65–3,570	1,260 ± 1,240	116–4,700	12,200 ± 9,010	1,600–34,000
		5 mo ($n = 14$)	1,270 ± 1,170	217–3,620	4,400 ± 3,200	450–10,600	27,900 ± 9,820	17,100–49,600
4	Nicotine (0–46 wks)/NNK	1–2 wks ($n = 24$)	1,900 ± 2,550	53–7,700	4,790 ± 5,860	70–17,500	18,900 ± 13,500	4,300–48,000
		5 mo ($n = 15$)	2,170 ± 2,760	156–10,500	5,700 ± 4,300	930–16,000	42,900 ± 19,400	16,600–74,400
5	Nicotine (0–2 wks)/NNK[a]	1–2 wks ($n = 25$)	980 ± 930	22–2,870	3,520 ± 6,220	150–3,120	17,000 ± 10,500	2,920–37,500
		5 mo	nd[d]		nd		nd	
6	Nicotine (2–46 wks)/NNK[b]	1–2 wks	nd		nd		nd	
		5 mo ($n = 20$)	840 ± 714	62–2,560	5,910 ± 4,140	820–14,700	37,800 ± 19,400	9,750–83,400

[a]Urine obtained from individual mice was analyzed by LC/MS-MS, values are provided for 2 time points for individual mice from which urine was collected.
[b]The protocol is as illustrated in Fig. 1: Beginning at 5 weeks of age, female A/J mice (groups 2, 4, and 5) were provided 0.44 μmol/mL nicotine in the drinking water; after 2 weeks, animals in groups 4, 5, and 6 were administered a single dose of NNK (10 μmol), nicotine was discontinued for group 5 and initiated in group 6 at the time of the NNK injection.
[c]Urine was collected from a single mouse between 7 and 14 days of nicotine consumption and the remaining samples were from a single collection per mouse from 2 to 7 days.
[d]nd, not determined, animals were not consuming nicotine at this time.

termination times, 18 days (96 hours after NNK or saline injection) and 46 weeks (Table 2). Plasma nicotine levels were low, typically less than 1 ng/mL. Nine animals in group 4, day 18, had plasma nicotine values greater than any animal in the other 2 groups, with the highest value equal to 11 ng/mL. It is unclear why the concentrations of cotinine and nicotine were higher in this group at this time of plasma collection. The mouse with the highest nicotine plasma value also was the animal with the highest cotinine and *trans*-3′-hydroxycotinine values, consistent with more recent and/or higher consumption of nicotine. As with the urine values, the variation in plasma nicotine concentrations was likely because of the time of last nicotine consumption. Although, circulating nicotine values were low at the single time points analyzed, the detection of nicotine concentrations as high as 11 ng/mL confirms that peak nicotine levels were at least 20 times higher.

O^6mG levels and P450 2A5 activity

To determine the effect of nicotine on the metabolic activation of NNK, O^6mG levels were quantified 96 hours following NNK treatment. A 96-hour time point was used, as a linear relationship between tumor multiplicity and lung DNA O^6mG levels exists at this time (33). There was no effect of nicotine consumption on O^6mG levels in the lung. The level of O^6mG in mice administered only NNK (group 3, $n = 10$) was 16 ± 5.7 pmol O^6mG/μmol guanine compared with 14 ± 1.9 for animals consuming nicotine throughout the experiment (group 4, $n = 10$). Consistent with this finding was the observation that nicotine consumption did not affect either lung or liver P450 2A5 activity. P450 2A5, a coumarin 7-hydroxylase, is a key catalyst of the metabolism of NNK to the reactive species that generates O^6mG (28). In hepatic microsomes from control mice (group 1), the rate of coumarin 7-hydroxylation was 6.4 ± 4.4 pmol/min/mg ($n = 10$), and in nicotine consuming mice (group 2), it was 5.4 ± 1.8 pmol/min/mg ($n = 10$). In pulmonary microsomes, the rates were 8.7 ± 3.9 pmol/min/mg ($n = 6$, group 1) and 7.7 ± 3.4 pmol/min/mg ($n = 6$, group 2).

Effect of nicotine on NNK-induced lung tumorigenesis

The administration of chronic nicotine in the drinking water did not result in an increase in lung tumor multiplicity or size relative to untreated animals (Table 3, groups 1 and 2). As expected, all groups administered a single dose of NNK had a significantly increased incidence of lung tumors, the average multiplicity was less than 1 in mice not treated with NNK and 18.4 to 21.9 in NNK-treated mice (Poisson analysis; $P < 0.0001$, Table 3). Similar differences were observed for tumor distribution. However, there was no significant difference in tumor multiplicity or the distribution of tumor numbers by size whether animals were consuming nicotine or not (group 3 vs. groups 4, 5, or 6). The concentrations of nicotine metabolites in plasma of individual animals were not significant variables when included in a regression model of the effect of group on tumor size and multiplicity distribution.

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Table 2. Plasma nicotine, cotinine, and *trans*-3′-hydroxycotinine concentrations

			Nicotine, ng/mL		Cotinine, ng/mL		*Trans*-3′-hydroxycotinine, ng/mL	
Group	Treatment[a]	Time	Mean	Range	Mean	Range	Mean	Range
2	Nicotine (0–46 wks)/ saline	Day 18 ($n = 20$)	0.61 ± 0.5	0.2–2.4	40 ± 45	7–198	34 ± 21	13–109
		Wk 46 ($n = 19$)	0.40 ± 0.46	0.1–1.9	19 ± 20	3–72	48 ± 36	6–111
4	Nicotine (0–46 wks)/ NNK	Day 19 ($n = 20$)	2.3 ± 1.5	0.3–11	118 ± 139	19–618	40 ± 32	6–120
		Wk 46 ($n = 18$)	0.66 ± 0.53	0.5–2.4	31 ± 23	12–84	45 ± 19	23–78
6	Nicotine (2–46 wks)/ NNK[a]	Day 18 ($n = 20$)	0.65 ± 0.76	0.1–2.5	32 ± 33	1–123	15 ± 14	0–45
		Wk 46 ($n = 20$)	0.26 ± 0.28	0.1–1.1	29 ± 19	6–53	62 ± 28	28–105

[a]The protocol is illustrated in Fig. 1 and described in Table 1. Mice in groups 2 and 4 were consuming nicotine in the drinking water from day 1 to the day of blood collection, animals in group 6 began consuming nicotine on day 14. Twenty mice in each group were euthanized on day 18 and the remaining mice (18–20) were euthanized at 46 weeks, the termination of the tumorigenesis protocol. Blood was collected immediately postmortem by cardiac venipuncture. Plasma was isolated and analyzed by LC/MS-MS for nicotine and its metabolites.

The tumors in the mice of each group were categorized as adenomas, adenomas with dysplasia, or carcinomas. The incidence (percent of mice with tumors) and the multiplicities of each tumor type are presented in Table 4. There was no significant difference in either incidence or multiplicity for any of the tumor categories between NNK-treated mice that consumed nicotine and those that did not (groups 4, 5, or 6 vs. group 3). There was a slightly higher but nonsignificant difference in carcinoma incidence in groups 4 and 6, versus group 3 or 5. However, there was no parallel decrease in either adenomas or adenomas with dysplasia, which is inconsistent with increased progression to carcinoma. On the basis of the data variance, we estimate that 80 mice per group would be required to detect a significant ($P < 0.05$) difference of this magnitude between these groups.

Discussion

Nicotine has been suggested to be a promoter of lung cancer. This suggestion is on the basis of numerous *in vitro* studies and several short-term xenograft studies (5–7, 11, 12). In the study presented here, we saw no effect of chronic nicotine consumption on the incidence, multiplicity, or progression of NNK-induced lung tumors in the A/J mouse. This was true whether nicotine was consumed before or after NNK administration. In this model system, which has been used extensively to study NNK carcinogenesis, nicotine did not affect either the initiation or progression of lung tumors. A recent study by Maier and colleagues also reported that nicotine administered in the drinking water had no effect on lung tumorigenesis in 2 additional mouse models (42).

Table 3. Effect of chronic nicotine consumption on NNK-induced lung tumor multiplicity and size in A/J mice[a]

				Tumors per mouse[b] (mean ± SD)			
				Total	Diameter, mm		
Group	*N*	Treatment[a]	Mice with tumors, %	<0.5	0.5–1.0	1.0–2.0	>2
1	19	No nicotine/saline	31	0.53 ± 0.1	0.05	0.32	0.11	0.05
2	19	Nicotine (0–46 wks)/saline	26	0.32 ± 0.1	0.00	0.21	0.05	0.05
3	18	No nicotine/NNK	100	18.4 ± 4.5	0.50	6.50	10.1	1.39
4	18	Nicotine (0–46 wks)/NNK	100	20.4 ± 5.4	0.28	7.89	11.3	0.94
5	19	Nicotine (0–2 wks)/NNK[b]	100	21.9 ± 5.3	0.80	7.90	11.8	1.40
6	20	Nicotine (2–46 wks)/NNK[b]	100	20.0 ± 5.4	0.55	5.80	12.3	1.30

[a]The protocol is illustrated in Fig. 1. Nicotine (0.44 μmol/mL) was administered in the drinking water for the time intervals indicated, and NNK (2 μmol) or saline was administered by intraperitoneal injection at 2 weeks. Twenty mice in each group were euthanized on day 18 for DNA adduct analysis, and the remaining mice (18–20) were euthanized at 46 weeks. One or two premature deaths occurred in groups 2 to 5, details are provided in Table 4. Because of a logistic error, only 19 animals were analyzed in group 1.
[b]The number of total tumors per mouse ranged from 6 to 41.

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Table 4. Histopathologic analysis of tumors in A/J mice receiving NNK with or without nicotine[a]

		Incidence (% of mice)			Multiplicity (average per mouse ± SD)		
Group	Treatment	Adenoma	Adenoma with dysplasia	Carcinoma	Adenoma	Adenoma with dysplasia	Carcinoma
1 ($n = 14$)	No nicotine/saline	7	7	0	0.07 ± 0.27	0.07 ± 0.27	0.0
2 ($n = 15$)	Nicotine (0–46 wks)/saline	8	0	15	0.08 ± 0.28	0.0	0.15 ± 0.40
3 ($n = 18$)	No nicotine/NNK	83	56	28	3.4 ± 2.4	0.83 ± 0.92	0.44 ± 0.78
4 ($n = 18$)	Nicotine (0–46 wks)/NNK	89	61	44	4.4 ± 2.7	1.0 ± 1.1	0.67 ± 0.91
5 ($n = 19$)	Nicotine (0–2 wks)/NNK	95	63	28	4.8 ± 3.5	1.0 ± 0.94	0.37 ± 0.68
6 ($n = 20$)	Nicotine (2–46 wks)/NNK	85	65	50	4.4 ± 2.6	0.8 ± 0.70	0.70 ± 0.80

[a]Treatments were as described in Fig. 1 and Table 3. Histologic analysis was not conducted on lungs from 5 mice in groups 1 and 2. Premature deaths occurred in groups 2 to 5 as follows: group 2, one death, week 3, cause of death—unknown, no microscopic analysis of tissues; group 3, one death, week 5, cause of death—unknown and one death at week 29, cause of death—disseminated lymphoma, pulmonary adenomas present; group 4, one death, week 43, cause of death—respiratory distress, adenocarcinoma with multifocal pulmonary adenomas, bronchiolar epithelial hyperplasia, alveolar histiocytosis, and hemorrhage and one death at week 37 after NNK injection. Cause of death—dorsal skin tumor (a large nonmalignant hematoma) pulmonary adenomas present; group 5, one death, week 11, euthanized because of "labored breathing" no microscopic analysis of tissues.

Nicotine metabolism in the mouse and in humans is qualitatively similar. Metabolism is catalyzed by closely related enzymes, P450 2A5 and P450 2A6, and the major metabolites in both species are cotinine and *trans*-3′-hydroxycotinine (25, 43). In smokers' urine, the proportion of nicotine to cotinine to *trans*-3′-hydroxycotinine is typically 1 to 1.5 to 4 (25). However, in the A/J mouse, the average proportions of these 3 compounds was 1 to 3 to 14, consistent with the greater catalytic efficiency of the mouse enzyme, P450 2A5, than its human ortholog CYP2A6 (26). A high efficiency of nicotine metabolism has also been reported in other mouse strains (43, 44).

As we initiated our study, Davis and colleagues reported that nicotine, administered as 3 weekly intraperitoneal injections [1 mg (4.8 μmol)/kg], increased both the number and size of NNK-induced tumors in the A/J mouse. This was a small study with a number of limitations. The weekly dose of nicotine used in their study, 0.36 μmol per mouse (assuming an average weight of 25 g) was almost 20-fold lower than the average weekly dose of nicotine consumed in our study, 6.6 μmol per mouse (15 mL/wk, 0.44 μmol nicotine/mL; Fig. 2). Administering nicotine by injection may result in a higher peak nicotine concentration than when nicotine is consumed in the drinking water. However, the mice would have experienced this nicotine concentration for less than 1 hour three days a week. No measure of plasma or urinary nicotine or cotinine was reported. The numbers of NNK-induced tumors reported for nicotine-treated and untreated animals were 16 ± 3 and 10 ± 3, $n = 8$ mice per group. This is a small number of mice for which to detect a significant difference in tumor number. In the A/J mouse, the variation in lung tumors per mouse is fairly large. In our study, it ranged from 6 to 41 in all NNK-treated mice ($n = 76$) and the SD for groups of 20 was 25%. In a recent study by Memmott and colleagues, which used a similar NNK dosing protocol to that used by Davis and colleagues, the number of lung tumors per mouse in NNK-treated animals ranged from 10 to 40 ($n = 15$; ref. 45).

The main limitation of our study is that because of the rapid metabolism of nicotine in the mouse, circulating nicotine levels were significantly lower than in smokers. However, the daily dose of nicotine per kg body weight is much higher than that of a smoker, and peak nicotine concentrations were at least 11 ng/mL, 20 times higher than circulating levels. The strength of the study is the chronic administration of nicotine and the length of administration. By following animals for 44 weeks after NNK administration, we were able to assess the progression of adenomas to adenocarcinomas. As noted in the results (Table 3), there was a nonsignificant increased number of carcinomas in groups 4 and 6 compared with groups 3 and 5. There was no parallel decrease in adenomas or adenomas with dysplasia, consistent with this observation being because of chance.

In summary, there were 3 key outcomes of our studies on the effect of nicotine on tumorigenesis in the A/J mouse, a species which is highly susceptible to lung tumors: (i) Chronic nicotine consumption alone did not induce lung tumors. (ii) Nicotine consumption up until the time of NNK administration did not decrease (or increase) tumor incidence. and (iii) Chronic nicotine consumption had no significant effect on tumor incidence, multiplicity, or progression. These negative findings in a commonly used model of human lung carcinogenesis should lead us to question the interpretation and relevance of *in vitro* and short-term xenograft studies of the effect of nicotine on cancer cells. Nicotine is a ligand for the nAChR and, therefore, increased exposure to nicotine will trigger many downstream changes but what

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the effect of chronic nicotine exposure is on these pathways in a smoker or ex-smoker is not clear.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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