In conclusion, the present experiments did not demonstrate any clear lung tumor promotion effects of thoracotomy or physical left lung collapse. It remains possible, however, that alternative approaches might have greater efficacy and these need more consideration. In addition, there was observed mesothelial cells reaction with the infused polymer. This infusion of certain materials directly to thoracic cavity with thoracotomy is easy operation and might be innovative method for the experiment like an animal pleural mesothelioma.

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Original Article

Toxicity of Nicotine by Repeated Intratracheal Instillation to F344 Rats

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Abstract: *In vivo*, nicotine in cigarette smoke induces various effects not only on the respiratory system but also the central and peripheral nerve systems, circulatory organs and digestive organs, and there is a possibility of promotion of lung tumorigenesis. The present experiment was conducted to examine histopathological changes caused by nicotine in the lung with repeated intratracheal instillation (i.t.). Six-week-old male F344 rats were administered nicotine by i.t. at doses of 0.05, 0.1 and 0.2 mg nicotine/rat every 3 weeks beginning at week 4, for up to a total of 9 times and were then sacrificed at week 30. The total number of administrations, total dose of nicotine and effective number of rats were 9 times, 0.45 mg and 5 rats and 4 times, 0.20 mg and 5 rats for the 0.05 mg nicotine/rat group; 3 times, 0.30 mg and 5 rats and 4 times, 0.40 mg and 3 rats for the 0.1 mg group; and 3 times, 0.60 mg and 3 rats for the 0.2 mg group, respectively. As a control group, 5 rats were administered 0.2 ml saline/rat 9 times. Some rats administered 0.1 and 0.2 mg nicotine suffered convulsions just after administration. Histopathologically, though proliferative changes were not observed, neutrophil infiltration, edema and fibrosis in the lung were induced by nicotine. In conclusion, repeated treatment of nicotine promoted neurologic symptoms in the acute phase, and strong inflammation in the lungs in the chronic phase, even at a low dose. Toxicity of nicotine is suggested to depend not on total dose of nicotine in the experiment but rather on repeated injury with consecutive administration. (DOI: 10.1293/tox.25.257; J Toxicol Pathol 2012; 25: 257–263)

Key words: nicotine, lung, intratracheal instillation, toxicity, rat

Introduction

There are many chemicals including carcinogens in cigarette smoke, and at least 4000 component compounds have been described[1]. Of the smoking-related chemicals, nicotine is one of the major important components with toxicity. Nicotine is taken into the blood via the lungs from the inhaled smoke and binds to nicotinic acetylcholine receptors on the central and peripheral nerves[2]. It thereby induces various effects not only in the respiratory system but also circulatory and digestive organs[3–5]. In addition, according to a previous report, nicotine enhances proliferation, migration, and radioresistance of human malignant glioma cells through EGFR activation[6]. Nicotine is the addictive component of tobacco acting on neuronal nicotinic receptors (nAChRs). Functional nAChRs are also present on endothelial, hematological and epithelial cells[7]. Nicotine has been shown to stimulate the growth of solid tumors *in vivo* and to promote gastric cancer in the stomach[8]. Tobacco carcinogens can initiate and promote tumorigenesis, so concomitant exposure to nicotine could confer a proliferative advantage to early tumors, although there is no evidence that nicotine itself provokes cancer[9].

However, there have only been few reports of *in vivo* toxicity and histopathological changes on aspiration of nicotine in the respiratory organs. To examine any lung toxicity induced by nicotine, it is necessary to have a system for frequent respiratory exposure. We have previously described a rat *in vivo* bioassay for detection of hazards due to fine particles by intratracheal instillation (i.t.)[10], which can be used for risk assessment of inhaled chemicals. The i.t. method has been proposed as the most reliable route for assessing the pulmonary toxicity of particles in rodents[11], although there are biologically different responses to inhalation and instillation[12,13]. Using this i.t. technique, the present experiment was conducted to histopathologically examine toxicity and cell proliferation caused by nicotine in the lungs by repeated i.t. administration *in vivo*.

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Materials and Methods

Chemicals

Nicotine (chemical formula: $C_{10}H_{14}N_2$ and CAS: 54-11-5) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and suspended in saline (Otsuka isotonic sodium chloride solution, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan).

Animals

Male Fischer-344/DuCrlCrj rats (4 weeks of age) purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) were maintained in the Division of Animal Experiments, Life Science Research Center, Kagawa University, according to the Institutional Regulations for Animal Experiments. The regulations included the best considerations for animal welfare and good practice of animal handling contributing to the replacement, refinement and reduction of animal testing (3Rs). The protocols of the experiments were approved by the Animal Care and Use Committee for Kagawa University. The animals were housed in polycarbonate cages with white wood chips for bedding and given free access to drinking water and a basal diet, CE-2 (CLEA Japan, Inc., Tokyo, Japan), under controlled conditions of humidity (60 ± 10%), lighting (12-h light/dark cycle) and temperature (24 ± 2°C). The experiments were started after a 2-week acclimation period.

Experimental design and tissue preparation

A total of thirty 6-week-old male F344 rats were randomly separated into 6 groups of 5 rats each and scheduled to be administered nicotine by i.t.[10] every 3 weeks from week 4 to 28, for a total of 9 times, and to be sacrificed at week 30. The doses of nicotine were decided to be 0.05, 0.1 and 0.2 mg nicotine/0.2 ml saline/rat based on the report that 0.2 mg nicotine/rat corresponds to a lethal dose for human adults (30–60 mg, 0.5–1.0 mg/kg body weight)[14,15]. The pHs of the diluted nicotine solutions (0.05, 0.1 and 0.2 mg nicotine/0.2 ml saline) were 9.22, 9.42 and 9.60, respectively (pH meter F-52, HORIBA, Ltd., Kyoto, Japan). The pH of the saline used as a vehicle and control was 6.14. During the experiment, the third or fourth administrations were not given to the high-dose group, 0.1 and 0.2 mg nicotine/rat, because of the death of some rats following nicotine administration. The final experiment protocol was therefore modified as shown in Table 1. The total numbers of administrations and effective numbers of rats were 9 times and 5 rats (Group 3) and 4 times and 5 rats (Group 2) for the 0.05 mg nicotine/rat group; 3 times and 5 rats (Group 4) and 4 times and 3 rats (Group 5) for the 0.1 mg group; and 3 times and 3 rats (Group 6) for the 0.2 mg group, respectively. As a control group (Group 1), 5 rats were administered 0.2 ml saline/rat 9 times. Total doses of nicotine per rat were 0.00 mg (Group 1), 0.20 (Group 2), 0.45 (Group 3), 0.30 (Group 4), 0.40 (Group 5) and 0.60 (Group 6).

At autopsy, the lungs, liver and kidneys were removed. The lungs were weighed including the trachea and heart first, rinsed in 10% neutral buffered formalin after excision and then infused with 10% neutral buffered formalin. The weights of the lungs were calculated by subtraction of the weight of the remaining trachea and heart. The liver and kidneys were weighed and immersed in 10% neutral buffered formalin for 3 days. Slices of organs were routinely processed for embedding in paraffin for histopathological examination of H&E-stained sections. For lungs, this was routinely performed for 2 slices of the left lobe and 1 slice each of the other lobes. Each lung lobe was examined histopathologically for neutrophil infiltration, pulmonary edema, pulmonary fibrosis, macrophage infiltration in the alveoli, restructuring of walls, granuloma[16] and atelectasis. Severity for each parameter, except for atelectasis, was divided as follows: 0, no change; 1, weak; 2, moderate; and 3, severe. Severity for atelectasis was divided as follows: 0, none; 1, 1 lobe; 2, 2 lobes; and 3, more than 3 lobes.

Statistical analysis

Body and organ weights were analyzed by the Tukey-Kramer post hoc test. P values less than 0.05 were considered significant.

Results

All rats in Groups 4, 5, and 6 (0.1 or 0.2 mg nicotine/rat) laid on their backs and suffered convulsions a few seconds after each i.t. administration. This symptom continued for approximately 5 to 10 seconds. At the third or fourth administration, there were a number of mortalities, although other rats survived the acute symptoms. As noted above, due to the deaths, the experimental design was modified, and the number of administrations was modified in some groups.

Final body and organ weights are summarized in Table 2. The body weights of the rats in Group 3 (0.05mg nicotine

Table 1. Details of the Final Experimental Protocol

Groups	Nicotine/rat (mg)	Total number of i.t.	Total dose of nicotine/rat (mg)	No. of rats	
				(Week 0)	(Week 30)
1	0.00 (saline)	9	0.00	5	5
2	0.05	4	0.20	5	5
3	0.05	9	0.45	5	5
4	0.10	3	0.30	5	5
5	0.10	4	0.40	5	3
6	0.20	3	0.60	5	3

9 times) showed significant decreases compared with Group 1 (control group). Absolute and relative weights of the lung were significantly increased in Groups 2, 3 and 5 compared with Group 1 (control group). Regarding liver weights, there were no significant differences compared with Group 1 (saline control group).

Histopathologically, the lungs of Groups 2–6 (nicotine-treated groups) showed inflammatory changes, neutrophil infiltration, pulmonary edema, pulmonary fibrosis, macrophage infiltration in the alveoli, restructuring of walls and granuloma, in all animals (incidence: 100%) (Table 3). Atelectasis was observed in Groups 2, 3, 5 and 6. No proliferative alteration of alveolar cells was apparent. The lungs of Group 3 (0.05 mg nicotine 9 times) showed the severest and most widespread inflammatory changes in all rats (Fig. 1-E and F). The inflammation in Groups 2, 4, 5 and 6 persisted until autopsy (week 30), despite the 17–20-week period between the final instillation of nicotine and autopsy. The areas of inflammation in the lungs of Groups 2, 4, 5 and 6 were localized and showed clear borders with normal areas (Fig. 1-C, Fig. 2-A, C, E).

All rats (Group 1-6) also showed severe lymphoid cell infiltration around the bronchus in their lungs with almost the same degree. The saline control group (Group 1) also featured severe lymphoid cell infiltration around the bronchus, but not inflammatory changes in the alveoli (Fig. 1-A, B).

In the kidneys and livers of animals treated with nicotine (Groups 2-6), no remarkable changes were observed macroscopically and histopathologically compared with the control group (Group 1).

Discussion

In the present study, rats treated with 0.1 or 0.2 mg nicotine suffered convulsions after each i.t. administration. The behavioral effects of nicotine are reported to be attributed to an action on nicotinic receptors, their over stimulation of nicotinic receptors in the brain resulting in clonic-tonic convulsions[17]. Damaj MI *et al.* reported that nicotine enhances the release of glutamate either directly or indirectly (membrane depolarization that opens L-type calcium channels) and that glutamate release in turn stimulates N-methyl-D-aspartate receptors, thus triggering a cascade of events leading to nitric oxide formation and possibly seizure[18]. Nicotine at a dose of 0.75 or 1.0 mg/kg body weight is reported to lead to a decrease in cortical after-discharge duration and influence seizure susceptibility, but not cause any detectable neuronal damage[19].

The body weights of the rats treated nicotine tended to be decreased compared with the control group (Group 1). Furthermore, in Group 3 (0.05 mg nicotine 9 times), the decrease was significant as compared with Group 1 (control group). This decrease in body weight can be considered due to the toxicity of nicotine. The total dose of nicotine in Group 3 was 0.45 mg and was lower than that in Group 6 (0.60 mg). However, the number of administrations in Group 3 was 9, and this was the maximum number. The results suggest that the decrease in body weight depends not only on the total dose of nicotine in the experiment but also on repeated and consecutive administrations. The lung weights of the rats treated with nicotine were increased significantly compared with the control group (Group 1) but not in the group treated with 0.1 mg nicotine 3 times (Group 4). This result corre-

Table 2. Body and Organ Weights of the Rats

Groups	Nicotine/ rat (mg)	Total number of i.t.	No. of rats	Body weight (g)	Liver		Lung	
					Absolute (g)	Relative (%)	Absolute (g)	Relative (%)
1	0.00 (saline)	9	5	341.6 ± 10.6[a]	9.7 ± 0.5	2.8 ± 0.1	2.0 ± 0.2	0.6 ± 0.1
2	0.05	4	5	314.6 ± 21.7	8.8 ± 0.5	2.8 ± 0.2	2.7 ± 0.2[b,c]	0.9 ± 0.1[b,c]
3	0.05	9	5	304.3 ± 24.5[b]	8.9 ± 0.6	2.9 ± 0.1	2.8 ± 0.4[b,c]	0.9 ± 0.2[b,c]
4	0.10	3	5	328.9 ± 13.0	8.6 ± 0.4	2.6 ± 0.1	1.6 ± 0.4	0.5 ± 0.1
5	0.10	4	3	335.7 ± 5.7	9.9 ± 0.8[c]	2.9 ± 0.3[c]	2.9 ± 0.3[b,c]	0.9 ± 0.1[b,c]
6	0.20	3	3	312.1 ± 7.9	8.8 ± 0.2	2.8 ± 0.0	2.6 ± 0.4[c]	0.8 ± 0.1[c]

[a] Average ± standard deviation. [b] $P<0.05$ vs. Group 1. [c] $P<0.05$ vs. Group 4.

Table 3. Scoring Indices of Histopathological Changes

Groups	Nicotine/ rat (mg)	Total number of i.t.	No. of rats	Neutrophil infiltration	Pulmonary edema	Pulmonary fibrosis	Macrophage infiltration in the alveoli	Restructuring of the walls of the alveoli	Granuloma	Atelectasis
1	0.00 (saline)	9	5	1.0 ± 0.4[a]	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.5	0.0 ± 0.0	1.0 ± 0.0	0.0 ± 0.0
2	0.05	4	5	2.4 ± 0.5	1.0 ± 0.0	1.2 ± 0.4	2.0 ± 0.0	1.4 ± 0.5	2.2 ± 0.5	1.6 ± 0.9
3	0.05	9	5	2.8 ± 0.4	1.6 ± 0.5	1.4 ± 0.5	2.2 ± 0.4	1.4 ± 0.5	2.8 ± 0.5	0.6 ± 0.9
4	0.10	3	5	2.4 ± 0.5	1.0 ± 0.0	1.0 ± 0.0	1.8 ± 0.4	1.0 ± 0.0	2.4 ± 0.0	0.0 ± 0.0
5	0.10	4	3	2.7 ± 0.6	1.7 ± 0.6	1.3 ± 0.6	1.7 ± 0.6	1.7 ± 0.6	2.7 ± 0.6	2.3 ± 0.6
6	0.20	3	3	2.3 ± 1.2	1.3 ± 0.6	1.3 ± 0.6	1.7 ± 0.6	1.3 ± 0.6	2.0 ± 0.6	2.0 ± 1.0

[a] Average ± standard deviation.

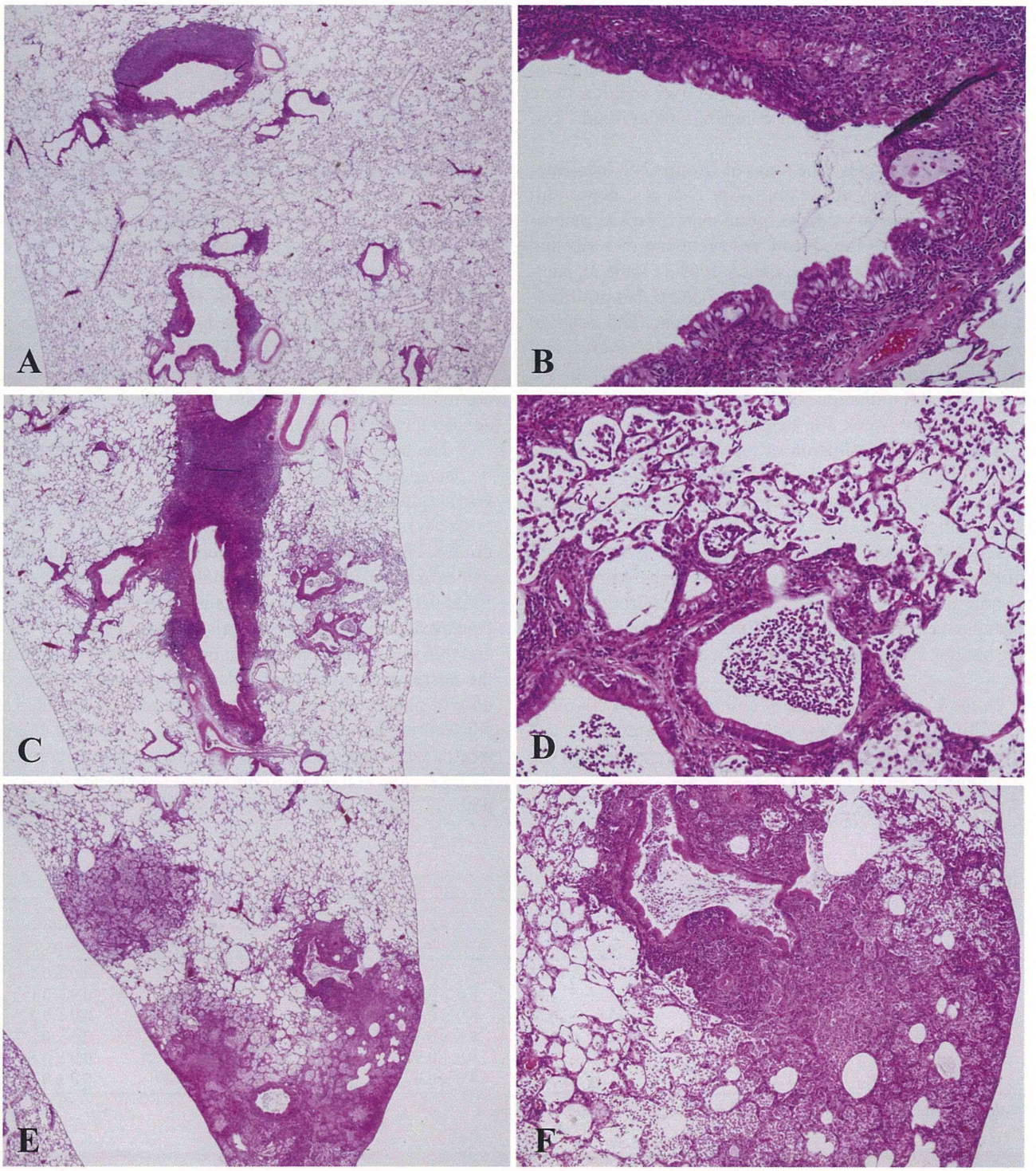

Fig. 1. Histopathological findings for lungs in Groups 1–3. The lungs of Group 3 (0.05 mg nicotine 9 times) showed the severest inflammatory changes in all rats (E and F). Inflammation in Group 2 (0.05 mg 4 times) persisted until autopsy (week 30). The saline control group (Group 1) also demonstrated severe lymphoid cell infiltration around the bronchus, as in the other groups (Group 2–6) (A and B). A, saline control ×9 (Group 1) (magnification: ×20); B, saline control ×9 (Group 1) (×200); C, 0.05 mg nicotine ×4 (group 2) (×12.5); D, 0.05 mg nicotine ×4 (group 2) (×100); E, 0.05 mg nicotine ×9 (group 3) (×12.5); F, 0.05 mg nicotine ×9 (group 3) (×40).

sponds with inflammatory change caused by nicotine in the lungs, excluding the decrease in lung weight in Group 4.

Histopathologically, the lungs of Group 3 (0.05 mg nicotine 9 times, 0.45 mg total dose) showed the severest and most widespread inflammatory changes in all rats. Histopathological inflammation also did not solely depend on the total dose of nicotine in the experiment, and repeated and consecutive administrations correspond with a de-

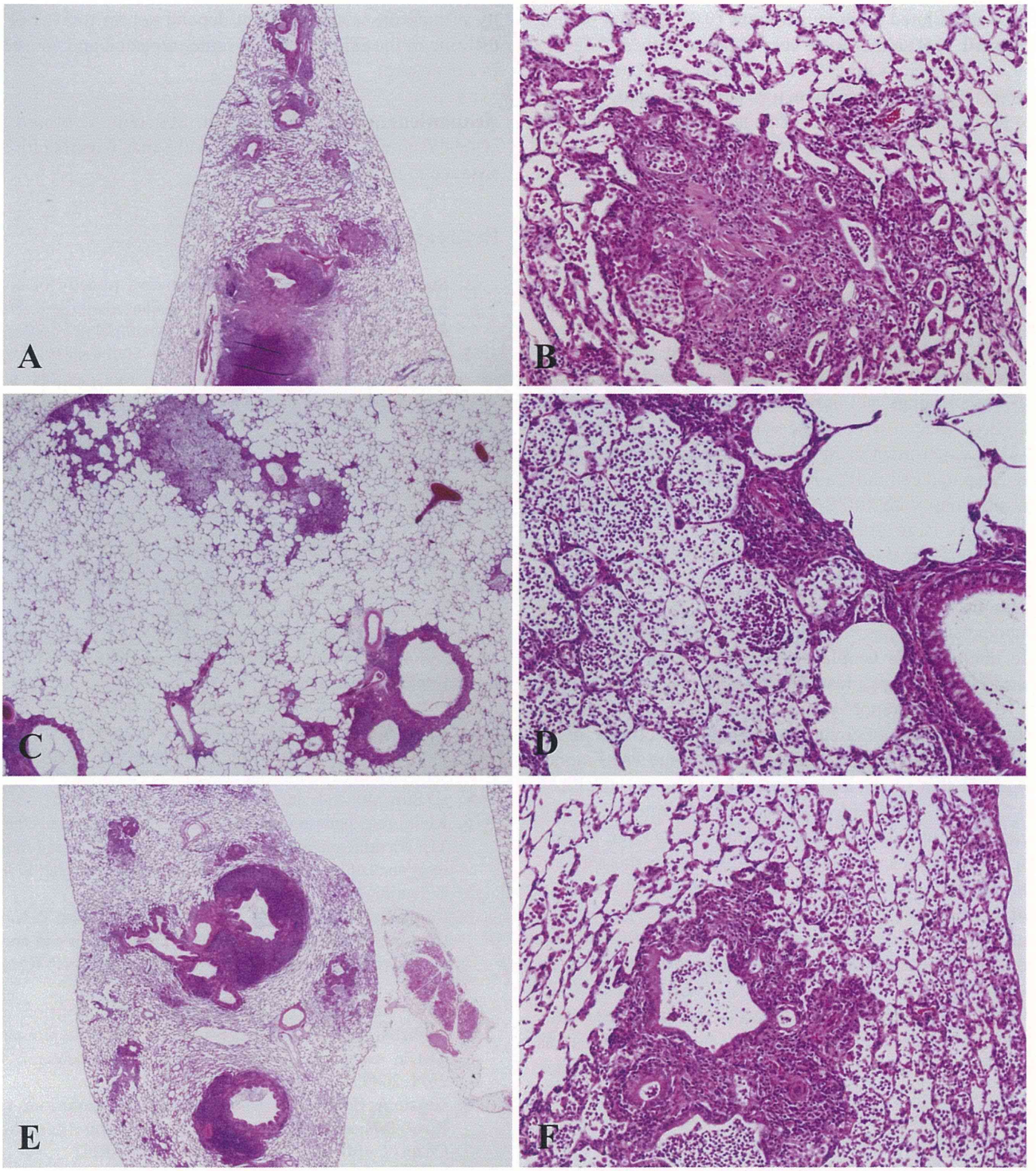

Fig. 2. Histopathological findings for lungs in Groups 4–6. Inflammation in Groups 4, 5 and 6 persisted until autopsy (week 30) with severe lymphoid cell infiltration around the bronchus (A–F). A, 0.10 mg nicotine ×3 (Group 4) (magnification: ×12.5); B, 0.10 mg nicotine ×3 (Group 4) (×100); C, 0.10 mg nicotine ×4 (Group 5) (×20); D, 0.10 mg nicotine ×4 (Group 5) (×100); E, 0.20 mg nicotine ×3 (Group 6) (×12.5); F, 0.20 mg nicotine ×3 (group 6) (×100).

crease in body weight. Mabley J *et al.* reported that intraperitoneal injection of 0.2 or 0.4 mg/kg nicotine exerts an anti-inflammatory effect in a murine model of acute lung injury induced by intratracheal lipopolysaccharide (LPS, 50 μl)[20]. The difference in the result of their report, exerting an anti-inflammation effect, and our experiment, inducing an inflammatory change, is suggested to be due to the difference in administration route. In the present experiment, the

pHs of the diluted nicotine solutions (0.05, 0.1 and 0.2 mg nicotine/0.2 ml saline) were very alkaline at 9.22, 9.42 and 9.60, respectively (saline: 6.14). Alkaline compounds cause liquefaction necrosis, which in turn causes ongoing invasion into deeper layers of tissue[21]. This is the same problem that occurs with accidental drinking of lye solution, the high pH of which is associated with esophageal ulceration. Vancula EM *et al.* concluded that the critical pH that causes esophageal ulceration is 12.5[21]. This is much higher than the solutions used in the present experiment. However, because the target organ is different, it is difficult to conclude that lung inflammatory changes were due to the nicotine itself or the high pH.

All rats (Groups 1–6) showed almost the same degree of severe lymphoid cell infiltration around the bronchus in their lungs, not only those treated with nicotine but also those treated with saline vehicle alone. Our previous 30-week experiment using F344 male rats also showed severe lymphoid cell infiltration around the trachea in a saline control group with 100% incidence, and this finding was reported as large granular lymphocytic lymphoma (LGL lymphoma)[22]. In this context, it should be mentioned that F344 rats demonstrate an incidence of over 50% of LGL lymphoma in aged animals[23]. Frith CH *et al.* concluded that lymphoid cell neoplasms in F344 rat should not be grouped with nonlymphoid neoplasms in determining the toxicity and carcinogenicity of test substances[24].

CYP2A5 is reported to be involved in metabolism of nicotine and its major circulating metabolite, cotinine, in the mouse liver[25]. CYP2A5, a mouse cytochrome P450 monooxygenase that shows high similarities to human CYP2A6 and CYP2A13 in protein sequence and substrate specificity, is expressed in multiple tissues, including the liver, kidney, lung and nasal mucosa. In humans, CYP2A6 is the predominant enzyme responsible for 70–80% of nicotine metabolism to cotinine[26,27]. The much higher exposure to cotinine than to nicotine in smokers should be taken into consideration, since cotinine suppression of apoptosis may play an important role in lung tumorigenesis *in vivo*[28]. We have established a bioassay using the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) as an initiating carcinogen[29]. In the lungs of A/J female mice, the initial event in this model is reported to be formation of O^6-methylguanine-DNA, a major promutagenic adduct that leads to GC>AT transitional mispairing and subsequent activation of the K-*ras* proto-oncogene[30,31]. We have previously reported inhibitory effects of 8-methoxypsoralen, a potent human CYP2A6 inhibitor, on NNK-induced lung carcinogenesis in female A/J mice[32,33]. Human CYP2A6 (mouse CYP2A5) might affect the metabolism of both nicotine and NNK. Therefore, it is strongly expected that human CYP2A6 inhibitors would have major effects on lung carcinogenesis after administration of nicotine and NNK.

In conclusion, repeated i.t. treatment with nicotine in the present study was associated with neurologic symptoms (convulsions) in the acute phase, and marked inflammation in the lungs in the chronic phase, even at a low dose. Toxicity of nicotine is suggested to depend not on total dose of nicotine in the experiment but rather repeated and consecutive exposure.

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Original Article

Lung tumorigenesis promoted by anti-apoptotic effects of cotinine, a nicotine metabolite through activation of PI3K/Akt pathway

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ABSTRACT — We previously found that genetic polymorphism in cytochrome P450 2A6 (CYP2A6) is one of the potential determinants of tobacco-related lung cancer risk. It has been reported that the plasma concentration of cotinine, a major metabolite of nicotine, in carriers of wild-type alleles of CYP2A6 is considerably higher than that in carriers of null or reduced-function alleles of CYP2A6, raising the possibility that cotinine plays an important role in the development of lung cancer. As a novel mechanism of lung tumorigenesis mediated by CYP2A6, we investigated the effects of cotinine on the suppression of apoptosis and promotion of lung tumor growth. In human lung adenocarcinoma A549 cells, cotinine inhibited doxorubicin-induced cell death by suppressing caspase-mediated apoptosis. Enhanced phosphorylation of Akt, a key factor responsible for cell survival and inhibition of apoptosis, was detected after cotinine treatment. These data suggest that cotinine suppresses caspase-mediated apoptosis induced by doxorubicin through activation of the PI3K/Akt pathway. Furthermore, we clarified that cotinine significantly facilitated tumor growth in the Lewis lung cancer model and accelerated development of lung adenomas induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in A/J mice. We herein propose that cotinine induces tumor promotion by inhibiting apoptosis and enhancing cellular proliferation, thus underlining the importance of CYP2A6 in tobacco-related lung tumorigenesis.

Key words: P450 2A6, Lung cancer, Tobacco smoking, Apoptosis, Nicotine

INTRODUCTION

Lung cancer is the most common form of cancer and is the leading cause of cancer-related mortality worldwide, causing approximately 1.2 million deaths annually (Jemal et al., 2009). Tobacco smoking is known to be the most important risk factor for lung cancer. Tobacco and tobacco smoke contain more than 60 carcinogens, such as N-nitrosamines, polycyclic aromatic hydrocarbons and aromatic amines (Hecht, 1998, 1999 and 2002). N-nitrosamines, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), require metabolic activation (mainly by cytochrome P450s (CYPs or P450s)) to exert their mutagenicity and carcinogenicity (Hecht, 1998). Many studies have been performed to identify the association between genetic polymorphisms of P450 genes and lung cancer risk (Le Marchand et al., 1998; Nakachi et al., 1991; Song et al., 2001).

We previously clarified that CYP2A6 is a major enzyme involved in the metabolic activation of NNK (Fujita and Kamataki, 2001; Kushida et al., 2000), and that null and reduced-function polymorphisms of CYP2A6 are significantly associated with lower risk of tobacco-related lung cancer (Ariyoshi et al., 2002; Fujieda et al., 2004; Miyamoto et al., 1999). In our hypothesis, subjects with reduced CYP2A6 capacity do not activate

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N-nitrosamines to the same extent as those with higher capacity. This hypothesis was confirmed by our previous finding that 8-methoxypsoralen, a potent inhibitor of CYP2A, completely inhibited the occurrence of NNK-induced adenoma in female A/J mice (Miyazaki et al., 2005; Takeuchi et al., 2003). CYP2A13, a CYP2A isoform that has 95.4% amino acid sequence identity to CYP2A6, was found to be expressed predominantly in the human respiratory tract. CYP2A13 has a higher potency to metabolically activate NNK than CYP2A6 does (Su et al., 2000), and CYP2A13*2 allele, Arg257Cys amino acid substitution, are reported to be associated with the reduction in risk of lung adenocarcinoma (Wang et al., 2003). On the other hand, a recent study has shown that various alleles of CYP2A13 including CYP2A13*1-CYP2A13*10 might have no association with risk of lung cancer in Japanese (Tamaki et al., 2011). These results were consistent with our previous data that it was no clear relationships between CYP2A13*2 allele and tobacco-related lung cancer risk in the previous epidemiology study (Fujieda et al., 2004). On the basis of discrepancy in these results of genetic polymorphisms in CYP2A13, it is difficult to rationalize our previous epidemiological finding by only CYP2A6-mediated metabolic activation of NNK. CYP2A6 is also responsible for the metabolism of nicotine, which is a major component of tobacco, to cotinine (Nakajima et al., 1996a, 1996b), and it has been reported that CYP2A6 genotypes are associated with different smoking behaviors (Fujieda et al., 2004; Pianezza et al., 1998; Thorgeirsson et al., 2010). Furthermore, the association of CYP2A6 with lung cancer risk was significant even when adjusting for smoking behavior (Fujieda et al., 2004). These lines of evidence suggest the possibility of unknown mechanism(s) related to CYP2A6.

It has been reported that nicotine and its derivatives, including NNK, activate the phosphatidylinositol 3'-kinase (PI3K)/Akt pathway and suppress apoptosis (Heeschen et al., 2001, 2002). We hypothesized that cotinine could exhibit tumor promotion effects, just as nicotine does, and could result in synergistic effects of NNK-induced initiation and cotinine-induced promotion of the development of lung cancer. In the present study, we examined the effects of cotinine on lung tumorigenesis using human lung adenocarcinoma cell lines and in vivo mouse models.

MATERIALS AND METHODS

Materials

NNK was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Cotinine was obtained from Sigma-Aldrich (St. Louis, MO, USA), and nicotine, doxorubicin and LY294002 were from Wako (Osaka, Japan). Phospho-Akt (Ser472), Akt antibodies and rabbit anti-goat IgG were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

Human lung adenocarcinoma-derived A549 cells were purchased from the American Tissue Culture Collection (Rockville, MD, USA). A549 cells were maintained in Dulbecco's modified Eagles medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (BioWhittaker, Walkersville, MD, USA), non-essential amino acids (Invitrogen, Carlsbad, CA, USA) and 1 mM sodium pyruvate (Invitrogen) at 37°C in 5% CO_2.

Animals

Female C57BL/6 and A/J mice (Japan SLC, Shizuoka, Japan) were maintained in the Graduate School of Pharmaceutical Sciences, Hokkaido University, according to the institutional animal care guidelines. All animals were housed in polycarbonate cages with white wood chips for bedding and were given free access to drinking water and a basal diet of Oriental MF (Oriental Yeast, Tokyo, Japan).

Cell viability assay and caspase-3/7 assay

One day before pre-treatment, cells were seeded at a density of 8×10^3 cells/well onto a 96-well plate. Cells were pre-treated with various concentrations (0.01-1 μM) of cotinine, nicotine (1 μM) or an equal volume of DMSO (as a vehicle control) in the absence or presence of LY294002 (20 μM), a PI3K inhibitor in FBS-free medium for 1 hr, and then 10 μM doxorubicin was added to this incubation. After incubation for 48 hr, cell viability was measured using Cell Proliferation Kit II (XTT) (Roche Diagnostics, Mannheim, Germany) or caspase-3/7 activities were measured using the Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA).

Immunoblotting

For dose-dependent induction of Akt phosphorylation, cells were incubated in 0.5% FBS-containing medium for 2 hr prior to treatment. Cells were then treated with cotinine (0.001-1 μM) for 45 min. When the effects of kinase inhibitors were examined, cells were pre-treated with LY294002 (20 μM) for 45 min prior to cotinine treatment. Cotinine was then added to this incubation and cultured for 45 min. The cell lysates were separated by SDS-PAGE and then transferred to polyvinylidene

fluoride membrane. After treatment with phospho-Akt (Ser473) or Akt antibodies (1:1,000 dilution), the protein-antibody complexes were detected by an ECL enhanced chemiluminescent detection system (Amersham, Arlington heights, IL, USA).

In situ TUNEL staining

Cells were seeded onto 100-mm^2 tissue culture plates in 10% FBS-containing medium. After 24 hr, the cells were pre-treated with cotinine (1 μM), nicotine (1 μM) or an equal volume of DMSO in FBS-free medium for 1 hr, and then treated with 10 μM doxorubicin for 24 hr. In situ DNA cleavage was assessed by the terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) reaction using a DeadEnd Colorimetric TUNEL System (Promega, Madison, WI, USA). Stained cells were observed using confocal microscopy (Olympus, Tokyo, Japan).

Lewis lung cancer model

Lewis lung carcinoma cells (1×10^6 cells/mouse) were subcutaneously injected into each flank of 10-week-old C57BL6 mice. Mice received cotinine (100 μg/ml in 2% saccharine), nicotine (100 μg/ml in 2% saccharine) or vehicle in the drinking water. There was no difference in water consumption between these test and control groups. At day 14, tumors were dissected from the mice. The tumor volume was calculated as the product of length × width × 0.5 cm^3.

In vivo tumorigenicity test

When female A/J mice were 7 weeks old, they were pre-treated with NNK (2 mg/ 0.1 ml saline/mouse, i.p.) or an equal volume of saline as a vehicle control. The next day, each group was given drinking water containing nicotine (100 μg/ml in 2% saccharine) (group 3), cotinine (100 and 300 μg/ml in 2% saccharine) (groups 4 and 5) or 2% saccharine alone as a vehicle control (group 2). The experiment was terminated 16 weeks after the first NNK treatment, and the surviving mice were killed under ether anesthesia. At autopsy, their lungs were excised and weighed, infused with 10% neutral buffered formalin and underwent careful gross inspection. All macroscopically detected lung nodules were counted, and each lung lobe was examined histopathologically. Lung lesions, hyperplasias and adenomas were diagnosed according to the criteria of "Tumors of the mouse" (Rehm et al., 1994). The number of adenomas was counted under a microscope.

Statistical analysis

One-way ANOVA followed by Dunnett's multiple comparison test was performed to examine the significance of differences in cell viability and caspase-3/7 activity. To evaluate the effects of LY294002, Student's t test was used. The data for multiplicity in A/J mice were also analyzed by Student's t test. All P values are two tailed, and the significance level was set at $P < 0.05$.

RESULTS

Effects of cotinine on doxorubicin-induced death of A549 cells

We investigated whether cotinine suppressed cell death induced by doxorubicin, a representative apoptosis inducer, using human lung adenocarcinoma-derived A549 cells. Survival of A549 cells decreased to 18.5% on treatment with 10 μM doxorubicin, whereas survival was reduced to 24.4%-40.0% and 45.0% on co-treatment with cotinine (0.01-1 μM) and doxorubicin (Fig. 1), and nicotine (1 μM) and doxorubicin, respectively, indicating that cotinine dose-dependently inhibited doxorubicin-induced cell death at a level almost equivalent to that of nicotine. The addition of LY294002, a PI3K-specific inhibitor, reduced the cotinine-elevated cell survival to 27.0%. In addition, we examined the effects of cotinine on the caspase-3/7 activation induced by doxorubicin (Fig. 1B). The caspase-3/7 activity in cells treated with doxorubicin was 4.4-fold higher than that of control. Cotinine repressed the doxorubicin-induced caspase-3/7 activation to 1.3- to 1.9-fold of control in a dose-dependent manner. To assess whether cotinine inhibits apoptosis, we then investigated the effects of cotinine on genomic DNA fragmentation, which is a hallmark of apoptosis. Although TUNEL-stained cells were not observed in control (Fig. 2A), treatment with doxorubicin gave rise to TUNEL-positive cells (arrowhead) and apoptotic bodies (arrow) (Fig. 2B). The number of TUNEL-positive cells was reduced in cells treated with doxorubicin and cotinine or doxorubicin and nicotine (Figs. 2C and 2D). Furthermore, cotinine blocked the genomic DNA fragmentation induced by doxorubicin (supplementary Fig. 1). These results suggest that cotinine suppresses apoptosis through the PI3K/Akt pathway just as nicotine does.

Effects of cotinine on phosphorylation of Akt

To confirm that cotinine activates the PI3K/Akt signaling pathway, we analyzed the phosphorylation level of Akt at Ser473. Cotinine enhanced Akt phosphorylation in a dose-dependent manner (Fig. 3A), whereas LY294002 inhibited the phosphorylation of Akt induced by cotinine

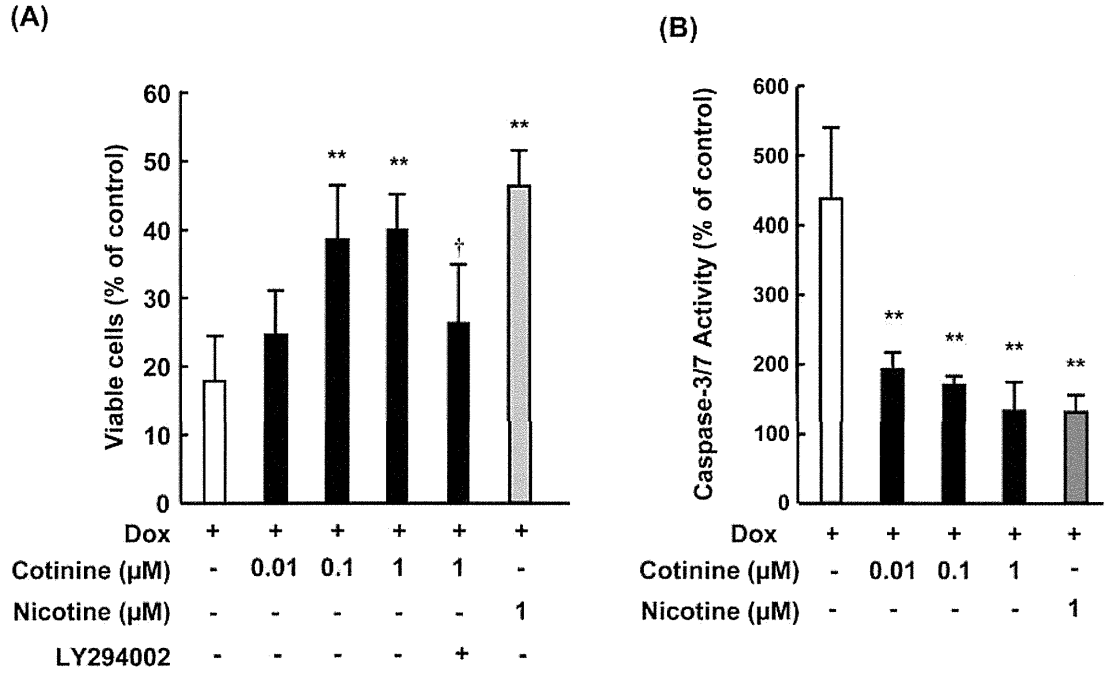

Fig. 1. Effects of cotinine on doxorubicin (Dox)-induced apoptotic death in A549 cells. (A) Cell viability. A549 cells were pre-treated with cotinine (0.01-1 μM) or nicotine (1 μM) in the presence or absence of LY294002 (20 μM), a PI3K inhibitor for 1 hr, and then treated with 10 μM doxorubicin for a further 48 hr. Cell viabilities were assessed by XTT assay. The percentage of viable cells relative to the vehicle control is given as the mean ± S.D. from three independent experiments. **Significantly different compared to doxorubicin alone at $P < 0.01$. †Significantly different compared to doxorubicin plus cotinine at $P < 0.05$. (B) Caspase 3/7 activity. A549 cells were pre-treated with cotinine (0.01-1 μM) or nicotine (1 μM) for 1 hr and then treated with 10 μM doxorubicin for a further 48 hr. Caspase-3/7 activities were measured using the Caspase-Glo 3/7 Assay Kit. Caspase-3/7 activities relative to vehicle control are given as means ± S.D. from three independent experiments. **Significantly different compared to doxorubicin alone at $P < 0.01$.

(Fig. 3B). These results indicate that cotinine induces Akt phosphorylation and suppresses apoptosis via the PI3K/Akt pathway.

Effects of cotinine on tumor growth in mice

On the basis of the above in vitro analyses, we evaluated whether cotinine enhanced tumor growth using the in vivo Lewis lung cancer model (Fig. 4). Two weeks after implantation of Lewis lung carcinoma cells and treatment with cotinine, tumor growth in the cotinine group significantly exceeded that in the vehicle-treated group by 2.3-fold ($P = 0.020$); nicotine-treated mice also showed significantly higher tumor growth than control ($P = 0.013$). We further examined the in vivo effects of cotinine on lung tumorigenesis induced by NNK in A/J mice (Table 1). Lung adenocarcinoma was not seen in any of the animals. The number of microscopically observed adenomas induced by NNK per mouse increased from 2.3 (NNK alone) to 2.7, 3.6 and 4.0 on treatment with 100 mg/l of nicotine, 100 mg/l of cotinine and 300 mg/l of cotinine, respectively; the increase in the group treated with 300 mg/l of cotinine compared to control group was statistically significant ($P < 0.05$). These results suggest that cotinine accelerates NNK-induced lung tumorigenesis.

DISCUSSION

In previous studies, we clearly demonstrated that reduced-function or null polymorphisms of CYP2A6 were significantly associated with lower risk of tobacco-related lung cancer (Ariyoshi et al., 2002; Fujieda et al., 2004; Miyamoto et al., 1999). The following two hypotheses to explain these associations were raised from the findings of several reports, including ours: first, differences in CYP2A6 activity caused by genetic polymorphisms result in different exposures to metabolically activated NNK,

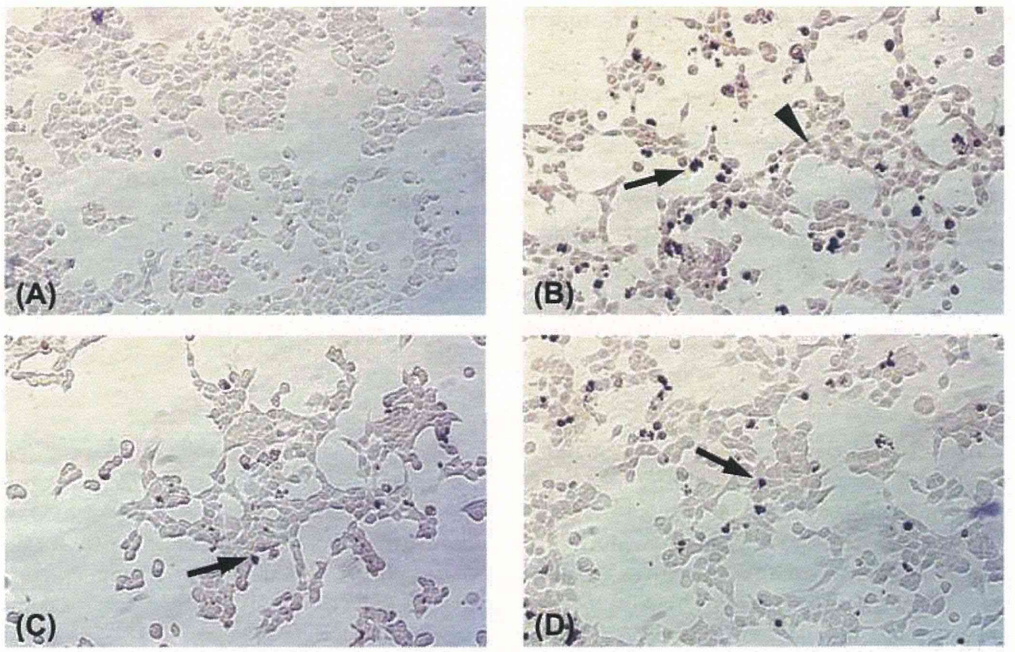

Fig. 2. Inhibitory effects of cotinine on genomic DNA fragmentation of A549 cells induced by doxorubicin. In situ TUNEL staining of control (A), and cells treated with doxorubicin alone (B), cotinine and doxorubicin (C), and nicotine and doxorubicin (D). A549 cells were pre-treated with cotinine (1 μM) or nicotine (1 μM) for 1 hr and then treated with doxorubicin (10 μM) for a further 24 hr. The arrowhead and arrows indicate TUNEL-positive nuclei (brown) and apoptotic bodies (dark brown), respectively. All panels display cells at 150 × magnification. Results are typical data found in three independent experiments.

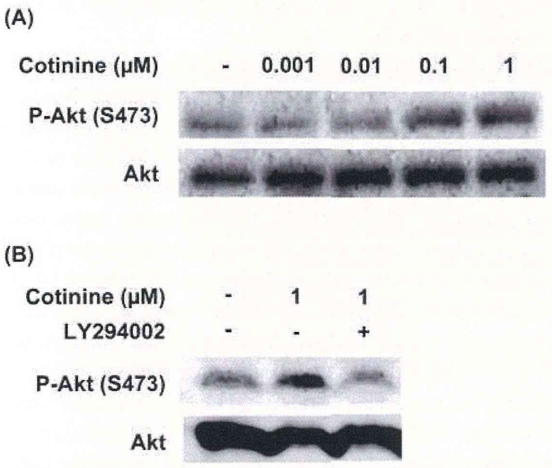

Fig. 3. Effects of cotinine on phosphorylation of Akt in A549 cells. (A) A549 cells were treated with cotinine (0.001-1 μM) in 0.5% FBS-containing DMEM for 45 min. (B) A549 cells were pre-treated with LY294002 (20 μM), a PI3K inhibitor for 45 min, and then treated with cotinine (1 μM) for a further 45 min. Phosphorylation of Akt was analyzed by immunoblotting using phospho-specific Ser473 antibody.

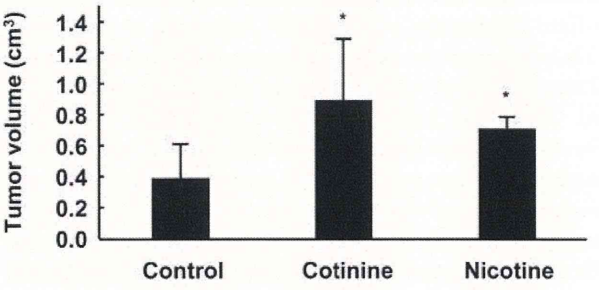

Fig. 4. Effects of cotinine on tumor growth in the Lewis lung cancer model. C57BL6 mice, subcutaneously injected with Lewis lung carcinoma cells (1×10^6 cells/mouse), received cotinine (100 μg/ml in 2% saccharine), nicotine (100 μg/ml in 2% saccharine) or vehicle in the drinking water. At day 14, tumors were dissected from the mice and the tumor volume was measured. *Significantly different compared to vehicle control at $P < 0.05$.

Table 1. Effects of cotinine on NNK-induced lung tumorigenesis in female A/J mice

Group	Treatment	Incidence (%)[a]	Tumors/mouse		
			Hyperplasia	Adenoma	Hyper+Adenoma
1	Saline alone	2/11 (18.2)	0.1 ± 0.3	0.1 ± 0.3	0.2 ± 0.4
2	NNK alone	12/12 (100)	0.5 ± 0.7	2.3 ± 0.9	2.8 ± 1.3
3	NNK + Nicotine 100 mg/l	12/12 (100)	1.3 ± 1.7	2.7 ± 1.6	4.0 ± 3.0
4	NNK + Cotinine 100 mg/l	11/11 (100)	0.9 ± 1.0	3.6 ± 1.7	4.5 ± 2.2
5	NNK + Cotinine 300 mg/l	12/12 (100)	0.9 ± 0.9	4.0 ± 1.3*	4.9 ± 1.2

[a]Number of mice observed with lung lesions.
*$P < 0.05$ vs Group 2.

and second, differences in CYP2A6 activity affects individual metabolism of nicotine leading to different smoking behaviors. However, the underlying mechanisms have not been fully clarified. In the present study, we propose a novel mechanism, namely that cotinine suppresses apoptosis and promotes tumor proliferation in vitro and in vivo. To our knowledge, this is the first study providing evidence that anti-apoptotic effects of cotinine play an important role in the development of lung cancer induced by tobacco smoking.

In humans, CYP2A6 is the predominant enzyme responsible for nicotine metabolism: nicotine is metabolized mainly (70%-80%) to cotinine by CYP2A6 (Nakajima et al., 1996a, 1996b), and is also metabolized to nornicotine by CYP2A6 and CYP2B6 via N-demethylation, which contributes to about 5% of nicotine metabolism (Yamanaka et al., 2005; Yamazaki et al., 1999). Therefore, genetic polymorphisms of CYP2A6 can cause large individual variations in nicotine metabolism. Xu et al. (2002) also reported that the plasma AUC of nicotine in subjects with lowered CYP2A6 activity was > 3-fold higher than that in those with normal CYP2A6 activity, suggesting that tumor promotion effects of nicotine in subjects of reduced CYP2A6 activity were greater than those in subjects of normal CYP2A6 activity. It appears to be disagreement with our hypothesis accounting for previous epidemiological results. However, some reports suggest that the pharmacokinetics of cotinine, a main metabolite of nicotine, was closely related with the genetic polymorphisms of CYP2A6. First, terminal half-life of cotinine (19 hr) was approximately 10-fold longer than that of nicotine (2 hr) in smokers (Benowitz et al., 1983). Second, the plasma AUC of cotinine in subjects with lowered CYP2A6 activity was 10-fold lower than that in those with normal CYP2A6 activity. The plasma AUC of both cotinine and nicotine in CYP2A6 extensive metabolizer was at least twice higher than that in CYP2A6 poor metabolizer (Xu et al., 2002). Third, urinary cumulative concentrations of cotinine in CYP2A6 extensive metabolizer were approximately 7-fold higher those in CYP2A6 poor metabolizer (Kitagawa et al., 1999). Finally, it has been reported that the mean plasma concentrations of nicotine and cotinine in smokers are 0.12 and 0.85 μM, respectively (Nagano et al., 2010). These lines of evidences clarified that the disposition of cotinine in smokers was greater than that of nicotine, suggesting that cotinine stimulates anti-apoptotic effects and tumor proliferative effects more than nicotine. In this study, we clarified that cotinine can suppress apoptosis at a level similar to that of nicotine. When the much higher expose to cotinine than to nicotine in smokers is taken into consideration, it is clear that cotinine may play an important role in lung tumorigenesis in vivo.

The PI3K/Akt pathway is a key mediator in regulating anti-apoptosis, cell survival and cell proliferation (Jimenez et al., 1998; Klippel et al., 1998). Active Akt has been detected in human lung cancer precursor lesions and in established lung cancers (Tsao et al., 2003). Non-small-cell lung cancer cells, including A549 cells, have constitutively active Akt that promotes cellular survival and resistance to chemotherapy or radiation (Brognard et al., 2001). This study demonstrated that cotinine exhibits anti-apoptotic and cellular proliferative effects through the PI3K/Akt-mediated signaling pathway. Nicotine induces phosphorylation of Bcl2 and Bad through mitogen-activated protein kinases (MAPKs) and the extracellular signal-regulated kinase (ERK1/2)-mediated signaling pathway as well as through the PI3K/Akt-mediated signaling pathway (Jin et al., 2004; Mai et al., 2003). In preliminary experiments, PD98059 (MAPK/ERK kinase-specific inhibitor) tended to block the cotinine-induced suppression of apoptotic cell death (data not shown) and phosphorylation of MAPK (supplementary Fig. 2), suggesting that cotinine is also likely to be involved in these

signaling pathways. We need to undertake further analyses to elucidate the detailed mechanisms. Because nicotine is reported to stimulate angiogenesis, we also investigated the angiogenic effects of cotinine, and clarified the cotinine-accelerated capillary formation of vascular endothelial cells as same level as those of nicotine (data not shown). A previous study reported that cotinine likely promoted the growth of vascular endothelial cells and vascular smooth muscle cells with a greater potency than that of nicotine (Carty et al., 1997), supporting the findings of our preliminary experiments. Therefore, cotinine may potentiate angiogenesis as well as suppress apoptosis, indicating that cotinine plays an important role in tobacco-related lung tumorigenesis.

In the present study, we investigated whether cotinine indicated anti-apoptotic effects and cellular proliferative effects using A549 cells, expressing nicotinic acetylcholine receptor (nAChR) and Bad (Jin et al., 2004; Plummer, 2005), but not CYP2A6/2A13 (Newland, 2011). It was reported that nicotine induced anti-apoptotic effects and angiogenesis effects via nAChR (Dwoskin et al., 1999), and improved cell survival rates by the phosphorylation of Bad through the PI3K/Akt or MAPK/extracellular signal-regulated kinase (ERK) pathway (Jin et al., 2004) (supplementary Figs. 2-4). Although it seemed to remain unsolved questions whether anti-apoptotic effects of cotinine could occur via nAChR and whether cotinine would have a higher affinity than nicotine, the affinity of cotinine to nAChR should be evaluated. We also examined the effects of cotinine and nicotine on the cell death using H82 (human small cell lung carcinoma) and H441 (human lung adenocarcinoma) in addition to A549 cells, which are pulmonary cancer cells (supplementary Fig. 4). Cotinine and nicotine significantly suppressed the doxorubicin-induced cell death in A549 cells, whereas the cellular proliferative effects of cotinine and/or nicotine were not significant in H82 and H441 cells. The cellular proliferative effects of cotinine and/or nicotine on doxorubicin-treated cells were not significant in H82 and H442 cells under the same conditions that the significant effects were observed in A549 cells. These differences may be due to the fact that Bad and nAChR are not expressed in H82 and H442 cells, respectively (Jin et al., 2004; Plummer, 2005). Furthermore, doxorubin was the most sensitive agents for detecting the cell death in A549 cells among the apoptotic agents including cisplatin and VP-16 (supplementary Fig. 5). Together with these findings, A549 cells treated with doxorubicin are likely to be an appropriate assay condition for evaluating the anti-apoptotic effects of cotinine.

In conclusion, we clarified that cotinine promotes lung tumorigenesis via suppression of apoptosis, giving more support to the impacts of genetic polymorphism of CYP2A6 on tobacco-related lung cancer risk. The novel mechanism of lung tumor promotion mediated by cotinine emphasizes the importance of CYP2A6 as a molecular target for chemoprevention against tobacco-related cancer in the lung.

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Gender-dependent effects of gonadectomy on lung carcinogenesis by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in female and male A/J mice

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Abstract. The present study was conducted to investigate the effects of gonadectomy on lung carcinogenesis in female and male mice, and to determine an association between sex hormone and lung carcinogenesis. Female and male A/J mice were divided into gonadectomized and unoperated control groups and all animals were treated intraperitoneally with 1 or 2 injections of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) at the dose of 2 mg/mouse. The mice were sacrificed 18 or 56 weeks after surgery. Serum levels of estradiol in females and testosterone in males were confirmed to be decreased by gonadectomy. Lung white nodules were detected in all mice of all groups. In the control groups of 18- and 56-week studies, the multiplicities of lung nodules in females were significantly greater than in males. In males in the 56-week study, the multiplicity of macroscopical lung nodules, bronchiolo-alveolar hyperplasias, adenomas and tumors (adenomas and adenocarcinomas) showed significant increase with castration. In females in the 18-week study, the multiplicity of adenomas decreased significantly by ovariectomy. Based on the results of the present study, female A/J mice were confirmed to be more susceptible to NNK-induced lung carcinogenesis than males. Furthermore, it was suggested that the process is inhibited by testosterone and accelerated by estradiol. These findings indicate the possibility that sex hormones play important roles in determining sex differences in lung carcinogenesis in the A/J mice initiated by NNK.

Introduction

Lung cancer prognosis remains very poor, with a 5-year survival rate generally <20%, lower than several other leading cancers, underlining the need for a better understanding of lung cancer formation and, ultimately, for identification of better therapeutic targets (1). Cigarette smoking is acknowledged to be the most important risk factor for human lung carcinogenesis and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a tobacco-specific N-nitrosamine which is considered to play important roles in tobacco-related human lung cancer (2,3). NNK is also a strong lung carcinogen in rodents, inducing bronchiolo-alveolar hyperplasia, adenoma and adenocarcinoma (4). Previously, we demonstrated that NNK-induced lung carcinogenesis in female A/J mice was strongly inhibited by treatment with 8-methoxypsoralen, a potent human cytochrome P450 2A6 (CYP2A6) inhibitor, before a single intraperitoneal injection (i.p.) of NNK (5-9). We also reported carcinogenic mechanisms and modifying effects of other factors using NNK-induced lung cancer animal models (10-15).

In the mouse, it is well known that females are generally more sensitive to chemical lung carcinogenesis, such as that induced by NNK, than males (16). It is hypothesized that the difference is due to difference in the amount of sex hormones such as testosterone and estradiol. Also, in humans, the incidence of adenocarcinoma type lung cancer is known to be generally higher in women than in men (17-19). The available data suggest that gender and sex hormones affect the character of lung cancer (20,21). For the purpose of these experiments, we hypothesized that the carcinogenesis of the lung may be influenced by sex hormone levels.

The present study was conducted to investigate effects of gonadectomy, ovariectomy and castration in female and male mice, respectively, on lung carcinogenesis and to determine associations with sex hormone levels, i.e. estradiol and testosterone. The experiments were conducted for 16 and 54 weeks after NNK treatment, since hyperplasia and adenoma lesions were earlier detected after 16 weeks (9), and adenocarcinomas were detected after 54 weeks (6).

Materials and methods

Chemicals. NNK was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada).

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Key words: lung tumorigenesis, gonadectomy, sex hormone, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, A/J mouse

Table I. Body and relative organ weights.

Group	Gender[a]	Treatment[a]	Duration[b]	No.[c]	Body weight[d] (g)	Lung[d] Relative (%)	Liver[d] Relative (%)	Kidneys[d] Relative (%)
1	F	NNKx2 OVX	18	21	24.81±2.80	0.70±0.08	4.50±0.26[e]	1.10±0.10
2	F	NNKx2 N	18	15	23.76±2.44	0.70±0.08	4.28±0.23	1.13±0.07
3	M	NNKx2 CAST	18	16	26.50±2.67	0.66±0.08	4.22±0.18[f]	0.98±0.05[g]
4	M	NNKx2 N	18	15	27.96±2.20	0.65±0.09	4.61±0.22	1.26±0.11
5	F	NNKx1 OVX	56	18	31.04±4.07[h]	1.23±0.53	4.20±0.52	1.57±2.08
6	F	NNKx1 N	56	12	25.80±3.09	1.27±0.23	4.18±0.76	1.19±0.27
7	M	NNKx1 CAST	56	21	33.19±4.17	0.86±0.21	3.67±0.23[i]	0.80±0.09[j]
8	M	NNKx1 N	56	14	30.33±4.41	0.86±0.18	4.28±0.40	1.34±0.13

[a]F, female; M, male; OVX, ovariectomy; CAST, castration; N, unoperated. [b]Weeks. [c]Number of mice examined. [d]Means ± standard deviation. [e]Significantly different from Group 2 by Student's t-test ($P<0.05$). [f]Significantly different from Group 4 by Student's t-test ($P<0.001$). [g]Significantly different from Group 4 by Welch's t-test ($P<0.001$). [h]Significantly different from Group 6 by Student's t-test ($P<0.05$). [i]Significantly different from Group 8 by Welch's t-test ($P<0.001$). [j]Significantly different from Group 8 by Student's t-test ($P<0.001$).

Animals. A total of 72 female and 72 male A/J mice (5 weeks of age), purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan), were maintained in the Division of Animal Experiments, Life Science Research Center, Kagawa University, according to the Institutional Regulations for Animal Experiments. The regulations included the best considerations on animal welfare and good practice of animal handling contributing to the replacement, refinement and reduction of animal testing (3Rs). The protocols of the experiments were approved by the Animal Care and Use Committee for Kagawa University. All animals were housed in polycarbonate cages with white wood chips for bedding, and given free access to drinking water and a basal diet, Oriental MF (Oriental Yeast Co., Ltd., Tokyo, Japan), under controlled conditions of humidity (60±10%), lighting (12 h light/dark cycle) and temperature (24±2˚C).

Experimental design. At 6 weeks of age, the mice were separated into 8 groups (Groups 1-8) of 15-21 animals each (Table I). Groups 1 and 5 were female groups undergoing ovariectomy. Groups 2 and 6 were females without ovariectomy. Groups 3 and 7 were male groups with castration, while Groups 4 and 8 were males without surgery. At the operations, the ovaries or the testes were removed under deep anesthesia (Groups 1, 3, 5 and 7) with i.p. of 0.025 ml pentobarbital sodium (Somunopentyl; Kyoritsu Seiyaku Co., Tokyo, Japan) diluted with 0.225 ml saline (Otsuka isotonic sodium chloride solution; Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan). The ovaries of female mice were resected from the retroperitoneum with a vertical incision in the back skin. The testes of male mice were resected from the center of the ventral scrota. The incision sites were closed with metal clips. At 2 weeks after surgery, all mice were treated with i.p. of NNK (2 mg/0.1 ml saline/mouse). After 3 weeks from surgery, Groups 1-4 were treated with another i.p. of NNK (2 mg/0.1 ml saline/mouse). The experiment was terminated after 18 weeks for Groups 1-4 and after 56 weeks for Groups 5-8. All surviving mice were sacrificed under deep anesthesia. Blood samples were collected from the mice in Groups 1-4 to measure the serum concentrations of estradiol and testosterone. These hematological examinations were performed at SRL Inc. (Tokyo, Japan). The detection limit of the estradiol was 10 pg/ml, and that of testosterone was 0.03 ng/ml. Values lower than these limits were considered as 0 and means for each group were calculated. At autopsy, the lungs, livers and kidneys were removed. The lungs were weighed including trachea and heart first, infused with 10% neutral buffered formalin after separation from the trachea and heart, and then immersed in 10% neutral buffered formalin. Lung weights were finally calculated by subtraction of the weight of the remaining trachea and heart. These procedures are appropriate for accurate weighing and good tissue preservation. After fixation in formalin, all lungs were carefully inspected grossly using a stereomicroscope. All macroscopically detected lung nodules were counted and trimmed for histopathological evaluation. The livers and kidneys were weighed and immersed in 10% neutral buffered formalin. Slices of lungs, livers, kidneys and macroscopic mass lesions were routinely processed for embedding in paraffin for histopathological examination of hematoxylin and eosin stained sections.

Histopathological analysis. Each lung lobe of all mice was examined histopathologically. Lung proliferative lesions were diagnosed as bronchiolo-alveolar hyperplasia, adenoma or adenocarcinoma according to the criteria of 'International Classification of Rodent Tumors: The Mouse' (22).

Statistical analysis. The incidences of lung proliferative lesions (macroscopically and histopathologically) were analyzed by the Fischer's exact probability test. Body and organ weights were analyzed by the Student's t-test or the Welch's t-test between groups. Multiplicities of lung proliferative lesions (macroscopically and histopathologically) were analyzed by the Student's t-test or the Welch's t-test between groups. The Student's t-test was applied when equal variance was obtained and the Welch's t-test with unequal variance.

Table II. Serum concentrations of estradiol and testosterone.

Group	Gender[a]	Treatment[a]	Duration[b]	Estradiol		Testosterone	
				No.[c]	(pg/ml)[d]	No.	(ng/ml)[d]
1	F	NNKx2 OVX	18	9	DL[e]	5	0.14±0.1
2	F	NNKx2 N	18	9	19.7±6.9	8	DL
3	M	NNKx2 CAST	18	9	DL	5	DL
4	M	NNKx2 N	18	12	DL	8	1.06±2.4

[a]F, female; M, male; OVX, ovariectomy; CAST, castration; N, unoperated. [b]Weeks. [c]Number of mice examined. [d]Means ± standard deviation. [e]DL, below the detection limit.

Table III. Incidences and multiplicities of macroscopical lung nodules.

Group	Gender[a]	Treatment[a]	Duration[b]	No.[c]	Macroscopical nodule	
					Incidence (%)	Multiplicity[d]
1	F	NNKx2 OVX	18	21	21/21 (100.0)	17.2±8.4
2	F	NNKx2 N	18	15	15/15 (100.0)	19.7±6.9[e]
3	M	NNKx2 CAST	18	16	16/16 (100.0)	14.3±6.2
4	M	NNKx2 N	18	15	15/15 (100.0)	12.2±5.7
5	F	NNKx1 OVX	56	18	18/18 (100.0)	18.2±7.6
6	F	NNKx1 N	56	12	12/12 (100.0)	20.6±6.0[f]
7	M	NNKx1 CAST	56	19	19/19 (100.0)	12.1±4.6[g]
8	M	NNKx1 N	56	14	14/14 (100.0)	7.6±3.3

[a]F, female; M, male; OVX, ovariectomy; CAST, castration; N, unoperated. [b]Weeks. [c]Number of mice examined. [d]Means ± standard deviation. [e]Significantly different from Group 4 by Welch's t-test ($P<0.05$). [f]Significantly different from Group 8 by Welch's t-test ($P<0.001$). [g]Significantly different from Group 8 by Student's t-test ($P<0.05$).

Results

General conditions. One mouse from Group 3 died 3 weeks after castration. In Group 5, one mouse was sacrificed after 25 weeks from ovariectomy due to poor general condition and another died 54 weeks after ovariectomy. In Group 6, 2 mice were sacrificed 4 and 17 weeks after surgery due to poor general condition and another mouse died 54 weeks after surgery. In addition, one mouse of Group 8 died 53 weeks after surgery. All other mice demonstrated no marked change in their general condition.

Body and organ weights. Body and organ weights are shown in Table I. In the 18-week study (Groups 1-4), body weights exhibited no significant variation across groups. In the 56-week study (Groups 5-8), body weights in Group 5 (ovariectomy group) were significantly increased as compared with Group 6. Lung weights showed no significant differences between ovariectomy or castration groups and each unoperated group. Liver weights of Group 1 (ovariectomy group) were significantly increased as compared with Group 2, while liver and kidney weights of Group 3 and 7 (castration groups) were significantly decreased as compared with Groups 4 and 8, respectively.

Sex hormones in the blood samples. Serum concentrations of estradiol and testosterone in each group in the 18-week study (Groups 1-4) are shown in Table II. Blood samples with quantities sufficient for estradiol measurement were obtained from 12 mice of Group 4, and from 9 mice of Groups 1, 2 and 3. For testosterone measurement, 5 mice from Groups 1 and 3, and 8 mice from Groups 2 and 4 were used. In ovariectomized females (Group 1), the estradiol concentration decreased to below the detection limit while the concentration of testosterone was slightly increased compared to Group 2, but without statistical significance. In castrated male mice (Group 3), the concentration of testosterone decreased to below the detection limit compared to Group 4.

Macroscopical analysis. Macroscopically, lung white nodules were detected in all mice of all groups (Fig. 1). In Group 7 (56-week study), 2 mice were excluded from macroscopical and histopathological analysis due to the failure in fixation of lungs. Nodules in the 56-week study (Groups 5-8) were clearly larger than those observed after 18 weeks (Groups 1-4). Data for incidences and multiplicities of macroscopical lung nodules are summarized in Table III. The incidences were 100% in all groups. In unoperated groups, the multiplicities of lung nodules were significantly greater in females than in