K-ras Mutations in Lung Tumors from NNK-treated Mice with Lipopolysaccharide-elicited Lung Inflammation

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Abstract. *Background: Chronic lung inflammation has been associated with an increased risk of lung cancer. However, it is unclear whether such an event affects the incidence of mutations in the K-ras oncogene frequently found in lung tumors and suggested to be involved in lung tumorigenesis. This study investigated potential impacts of inflammation on the incidence of lung tumors and K-ras mutations using a mouse model. Materials and Methods: FVB/N mice were treated with lipopolysaccharide (LPS) for 16 weeks with or without co-treatment with 4-(methyl-nitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) during the first 4 weeks. Results: There was a significant increase in lung inflammatory responses in mice treated with LPS and with LPS+NNK, compared with mice treated with NNK or with vehicle. The average number of lung tumors per mouse was 3.87 (between 1 and 6) and 0.73 (between 0 and 3) in mice treated with LPS+NNK and NNK alone, respectively (p<0.0001). No lung tumors were observed in mice treated with LPS or vehicle. A higher proportion of lung tumors from mice treated with LPS+NNK had K-ras mutations, compared with the mice treated with NNK alone (81.03% versus 45.45%, p<0.05). Conclusion: LPS-elicited chronic lung inflammation significantly increases the risk of NNK-mediated lung tumorigenesis in FVB/N mice through K-ras gene activation by point mutations.*

Lung cancer, the leading cause of cancer death in the United States and worldwide (1, 2), is a complex disease that develops through a series of sequential genetic and epigenetic alterations in oncogenes and tumor suppressor genes, causing subtle alterations in growth control (3-5). Epidemiologic data strongly associate exposure to exogenous factors, chiefly tobacco smoking, with increased risk of lung cancer as some of the molecular changes in lung tumors are similar to those caused by genotoxic carcinogens found in tobacco smoke (6, 7).

Aside from direct carcinogens, inhaled smoke contains other constituents that can stimulate chronic lung inflammatory responses (8-10) and these events have been associated with an increased lung cancer risk. For instance, lung diseases, including chronic obstructive pulmonary disease, have been shown to be an independent predictor of lung cancer risk (11-15). Other studies have attempted to gain insight into the mechanisms of inflammation-mediated increased risk of lung cancer. It has been shown that the transcription factor, nuclear factor kappa beta (NK-κB), and other genes play an important role in the promotion, growth, and metastasis of lung tumors (16-24).

Our previous studies demonstrated that mutations in the *K-ras* oncogene occurred in a high proportion of lung tumors from mice treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a potent lung carcinogen found in tobacco smoke, and these mutations have been suggested as early events in lung tumorigenesis (25). It is currently unclear whether chronic lung inflammation may affect the incidence of lung tumors and/or the frequency of *K-ras* mutations in these tumors in a mouse model.

In this study, we used a mouse model for treatment with the known lung carcinogen, NNK, in the presence and absence of pulmonary inflammation elicited by exposure to lipopolysaccaharide (LPS), a major pro-inflammatory glycolipid component of the gram-negative bacterial cell wall (8-10). We compared results on inflammatory responses, lung tumor incidence, and types and frequencies of mutations of the *K-ras* gene between mice treated with NNK alone and those treated with LPS and NNK combined.

Materials and Methods

Materials. Seven-week-old FVB/N mice, both male and female, were used in this study. They were originally purchased from Jacksons Laboratory (Bar Harbor, Maine, USA). Mice were house-

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inbred and maintained in accordance with the current guidelines for animal welfare in the Guide for the Care and Use of Laboratory Animals, 1996, Animal Welfare Act, 1996, as amended in 1970, 1976 and 1985, 9 CFR Parts 1,2,3.

The following materials were used: lipopolysaccharide (LPS, L8643; Sigma, St. Louis, MO, USA); 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK; Toronto Research Chemicals, Inc., North York, ON, Canada).

Chemical administration. Mice were separated into four groups (groups 1 to 4, each of 15 animals). Treatment was carried out as follows: for group 1, each mouse was treated with LPS for 16 weeks and with NNK for the first 4 weeks. For LPS treatment, on the first day of each week mice were first put under light anesthesia by intraperitoneal (*i.p.*) injection with Avertin (50 mg/kg of body weight), then intranasally instilled with 5 μg of LPS in 30 μl sterile phosphate-buffered saline (PBS). For NNK treatment, during each of the first 4 weeks, each mouse was administered with two *i.p.*injections (each consisting of 3 mg NNK in 200 μl saline) on day one and day three following LPS treatment. Mice in group 2 were treated only with LPS for 16 weeks, while mice in group 3 were treated only with NNK for the first 4 weeks. In addition, saline injection was used for mice in group 2 in place of the NNK injections, and for mice in group 3 in place of LPS. For control group 4, mice were injected with saline for 16 weeks. Mice were monitored regularly until lungs were harvested one week after the last LPS injection.

Bronchoalveolar lavage fluid (BALF) preparation, lung tumors and K-ras mutation analysis. For BALF and cell differential counts, at 17 weeks after the initial LPS treatment, mice were each anesthetized by *i.p.* injection with 2.5% Avertin and exsanguinated. The trachea was cannulated, and the lungs lavaged with 1 mL PBS, and the BALF samples pooled (*pool 1*). The lungs were lavaged an additional five times with 1 mL PBS and the recovered fluid pooled (*pool 2*). Cells from the two pools were recovered through centrifugation at 300 × g and resuspended in 0.5 mL PBS. A 50 μL aliquot was stained with an equal volume of 0.4% trypan blue (Invitrogen, San Diego, CA, USA) and cells counted with a hemocytometer. An additional aliquot was placed onto glass microscope slides (Shandon Cytospin 4; Thermo Fisher, Pittsburgh, PA, USA), stained with Diff-Quick, and cell differential was determined microscopically.

Each lung was perfused with 4% paraformaldehyde and paraffin-embedded. Successive 5 μm-thick sections were prepared, stained with hematoxylin and eosin, and histologically examined. Lung tumors were isolated by laser capture microdissection and deparaffinized from lung tissue sections. DNA was recovered by treatment with proteinase K, phenol/chloroform extraction and ethanol precipitation.

K-ras mutations were analyzed as described previously (25) by using the combination of nested PCR to amplify *K-ras* exon 1, which contains codon 12/13, and denaturing gradient gel electrophoresis (DGGE) to separate mutant from wild-type alleles. Briefly, for the first PCR, an aliquot containingthe equivalent of 50-100 cells was taken from each DNA sample and used for amplification in a final 25 μL volume containing 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 100 μmol/L each deoxynucleotide triphosphate, 0.3 μmol/L of each primers P1(sense) 5'-GACATGTTCTAATTTAGTTG-3' and P2 (antisense) 5'-AGGCGCTCTTGCCTACGGCA-3'), and 1.0 unit AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). The mixture was heated at 95°C for 10 min and then
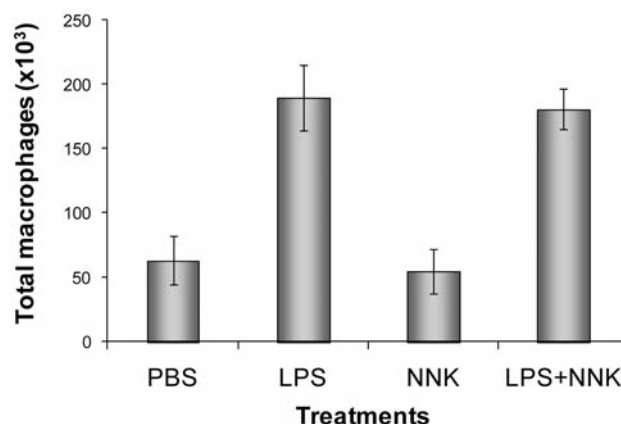
subjected to 40 cycles (94°C for 1 min, 58°C for 1 min, and 72°C for 1 min). For the secondPCR, 1 μL of each of the first-round PCR products was diluted into a final 25 μL reaction mixture as above, except that 0.25 μL [α-^{32}P]dATP (3,000 Ci/mmol; Perkin-Elmer, Waltham, MA, USA) was added and that primers P2 (antisense) and P3(sense) 5'-GCCGCCTGCAGCCCGCGCCCCCCGTGCCCCCG CCCCGCCGCCGGCCCGGCCGCCTATTGTAAGGCCTGCTGAAA AT-3' were used to generate a PCR-fragment containing *K-ras* gene exon 1 with a high temperature melting domain needed for DGGE analysis (25). Each reaction mixture was heated at 95°C for 10 min and subjected to 25 cycles (94°C for 1 min, 60°C for 1 min, and 72°C for 1 min). The resulting PCR products were separated by polyacrylamide gel electrophoresis and autoradiographed. Bands containing the expected 126-bp exon 1 fragment were excised from the gel. DNA was eluted and analyzed by denaturing gradient gel electrophoresis (Bio-Rad, Hercules, CA, USA) under the conditions described previouslyand mutant alleles were further characterized by sequencing (25).

Statistical analysis. Fisher's exact test or Chi-Square (x^2) test was used to test the difference in tumor numbers and *K-ras* mutation frequencies between the different groups of mice. A *p*-value of 0.05 was considered statistically significant. Analysis was performed using the STATA 9.2 software for Windows (StataCorp LP, College Station, TX, USA).

Results

Analysis of inflammatory responses in BALF and lung tissue sections. BALF samples obtained from groups of mice treated with LPS alone and mice treated with LPS+NNK showed a high number of macrophages and neutrophils, compared with mice treated with NNK alone and those treated with saline. There was an approximate 3-fold increase in the total number of macrophages in the BALF of mice treated with LPS alone, compared with the mice treated with saline (Figure 1). There was also an approximate 3-fold increase in macrophages in BALF of the mice treated with LPS+NNK, compared with those treated with NNK alone. There was no difference in the frequencies of
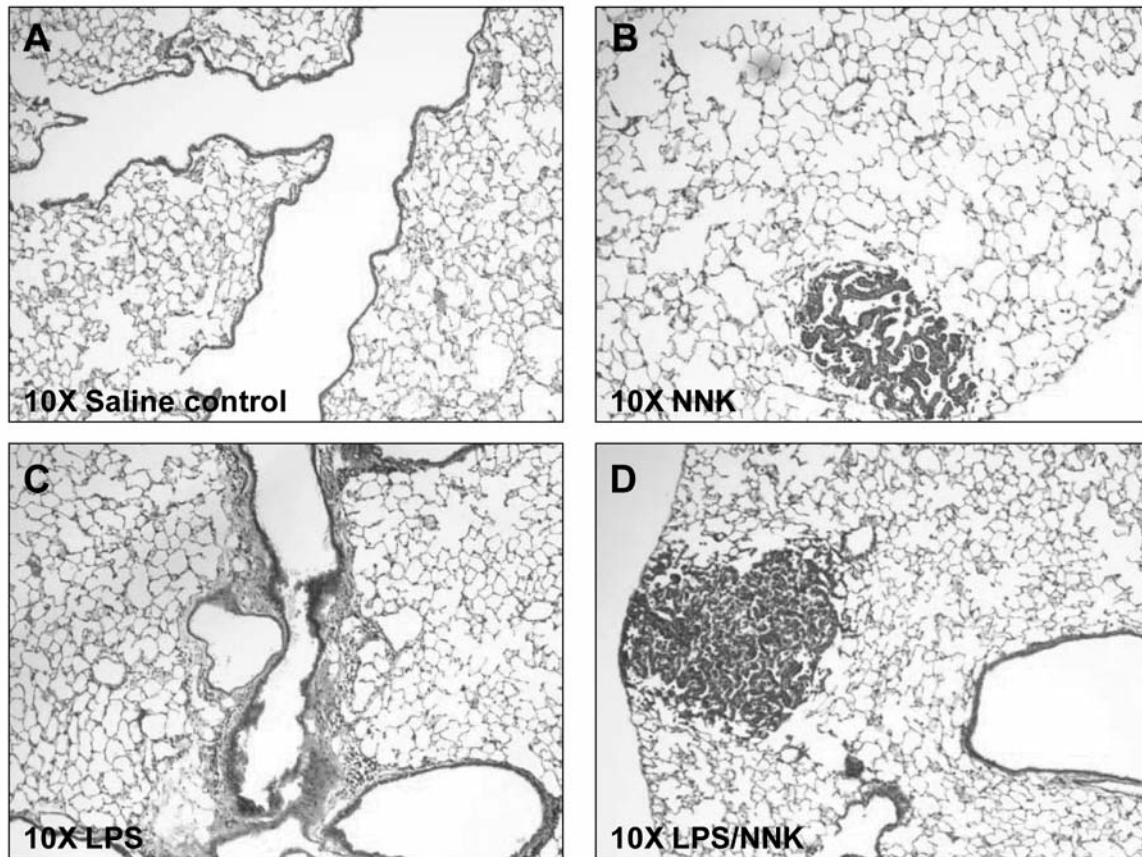

Figure 1. *Total number of alveolar macrophages in BALF of mice treated with saline, LPS, NNK, and LPS+NNK.*

Figure 2. *Histopathologic analysis of mouse lung tissue sections. Paraffin-embedded lung tissue sections were made from saline-treated (A), NNK-treated (B), LPS-treated (C), and LPS- and NNK-treated (D) mice sacrificed at week 17 and then stained with hematoxylin and eosin.*

macrophages or neutrophils between the NNK- and saline-treated groups, suggesting that NNK alone did not induce a detectable change in inflammatory cells in BALF. These data are in agreement with a previous study showing that LPS caused strong inflammatory responses detectable in BALF (10).

Pathological examination of lung tissue sections stained with hematoxylin and eosin prepared from saline-treated mice sacrificed one week following treatment showed histopathologically normal lung tissues without macrophages or dysplasia (Figure 2A). For comparison, injured tissue and inflammatory cells were observed in the LPS-treated mice (Figure 2C). Similar observations were made for the mice treated with LPS+NNK (Figure 2D), although the extent of inflammation was less severe, compared with the mice treated with LPS alone. The NNK-treated group showed no inflammation and macrophages were not increased when compared with the PBS control group (Figures 2A and 2B, respectively). These results indicate that LPS induced inflammation in the lungs of mice treated with LPS alone or in combination with NNK. Treatment with NNK alone did not induce any significant lung inflammation, compared with

the saline-treated mice. Results of pathological analysis of lung tissue sections obtained from saline-treated mice sacrificed 17 weeks after initial treatment showed normal lung tissue. For comparison, the LPS-treated mice showed some hyperplasia and mainly perivascular inflammation but no tumors. The NNK-treated and the LPS+NNK-treated groups showed adenoma and adenocarcinoma in some lungs and injury in parenchyma in some others. Taken together, treatment of mice with LPS and LPS+NNK led to lung tissue inflammation and injury as revealed by both pathological analysis of lung tissues and increased concentrations of inflammatory cells and mediators in BALF. These results are in agreement with those reported by a previous study (10).

Analysis of lung tumors and K-ras mutations. As shown in Table I, no tumors were detected among the mice treated with saline or those treated with LPS alone. For comparison, 7 out of the 15 mice treated with NNK developed lung tumors, including one mouse with three tumors, two mice each with two tumors, and four mice each with one tumor. Therefore, a total of 11 lung tumors were found among the 15 NNK-treated mice,

Table I. *Tumor multiplicity in NNK-treated and LPS+NNK-treated mice.*

Number of tumors per mouse	Number of mice with tumor(s)	
	NNK	LPS+NNK
0	8	0
1	4	1
2	2	2
3	1	0
4	0	8
5	0	3
6	0	1
Total tumors number	11	58

Table II. *Size of lung tumors and K-ras mutations in NNK-treated and LPS+NNK-treated mice.*

Tumor size (diameter in mm)	NNK		LPS+NNK	
	Tumor number	*K-ras* mutations	Tumor number	*K-ras* mutations
0.3 or less	3	2	26	26
0.3-0.7	3	2	25	17
0.7 and higher	5	1	7	4
Total	11	5	58	47
K-ras mutation rate	45.45%		81.03%	

resulting in an average of 0.73 tumors per mouse. Furthermore, all mice treated with LPS+NNK developed lung tumors, including one mouse with 6 tumors, three mice each with 5 tumors, eight mice each with 4 tumors, two mice each with 2 tumors, and one mouse with 1 tumor. A total of 58 tumors were found among 15 mice, or an average of 3.87 tumors per mouse. Therefore, co-treatment with LPS results in a 5.27-fold increase in the number of NNK-mediated lung tumors per mouse, compared with the group treated with NNK alone.

The diameters of the tumors ranged from approximately 0.1 mm to 1.1 mm and can be separated into three different size groups in both the NNK-treated group and the group treated with LPS and NNK (Table II). There was no significant difference regarding tumor size between mice treated with NNK or LPS+NNK. Although the number of tumors in the NNK-treated group was relatively small in our study, our results on the range of tumor size were in agreement with those reported previously (26, 27).

K-ras mutations were identified in 5 (45.45%) of the 11 tumors from the NNK-treated mice. For comparison, 47 (81.03%) of the 58 tumors from the mice treated with LPS+NNK each had a *K-ras* mutation. Therefore, there is a significantly higher incidence of *K-ras* mutation in lung tumors from mice treated with LPS+NNK, compared with mice treated with NNK alone ($p<0.05$). All mutations were detected in codon 12 of the *K-ras* gene and consisted of a GGT (glycine) to GAT (aspartate) transition, except for four tumors from the group of mice treated with LPS+NNK, which showed a GGT to GTT (valine) or TGT (cysteine) transversion.

Discussion

In this study, we have applied a mouse model for treatment with the lung carcinogen NNK to investigate the effects of LPS-elicited chronic pulmonary inflammation on lung cancer risk. Results from the group of NNK-treated FVB/N mice show that the incidence of lung tumors (46.67%) and the

multiplicity (0.73 tumors per mouse), as well as the rate of *K-ras* mutations (45.45%) in these tumors, are in agreement with our previous studies using the same strain of mice and treatment conditions (25).

We observed that the treatment of mice with LPS alone led to high inflammatory responses but did not result in any change in lung tumor incidence compared with mice treated with saline. Nevertheless, co-treatment of mice with LPS and NNK resulted in at least a 5-fold increase in the number of lung tumors per mouse compared with mice treated with NNK alone (3.87 *versus* 0.73, $p<0.0001$).

The increased incidence of lung tumors in mice treated with LPS+NNK resulted in an increased tumor multiplicity in this group, compared with the group treated with NNK alone. Furthermore, while there was no significant difference in tumor size between the NNK-treated group and the group treated with LPS+NNK, our results suggest an association between the presence of *K-ras* mutations and tumor size within each group. For instance, in mice treated with LPS and NNK, tumors harboring *K-ras* mutations had an average diameter size of 0.363 mm (SD±0.139) and were significantly smaller than tumors without *K-ras* mutations that had an average size of 0.476 (SD±0.201) ($p=0.027$). Moreover, in the mice treated with NNK alone, tumors with *K-ras* mutations were slightly smaller, compared with tumors without these mutations, but the difference was not statistically significant, which is probably due to the smaller number of tumors in the NNK-treated group (0.420±0.134 mm *versus* 0.438±0.226 mm) ($p=0.936$). The reasons for the size difference between tumors with *K-ras* mutations and those without these mutations are unclear. A previous study of mice treated with *N*-nitrosodimethylamine showed that lung carcinomas with mutant *K-ras* were significantly smaller than those lacking mutations, (26). Furthermore, an earlier study of human lung tumors reported that tumors with mutant *K-ras* were smaller, less differentiated and had a poorer prognosis compared with tumors with wild-type *K-ras* (27).

It is unclear how LPS-elicited pulmonary inflammation led to an increased lung tumor incidence in our study. Some studies suggested that alveolar macrophages play animportant role in mediating the effects of LPS that enters the lungs. During the earliest event in LPS-induced inflammation, LPS is transferred to its cellular receptor complex formed between toll-like-receptor-4, pattern recognition receptor CD14, myeloid differentiation-2, and LPS-binding protein, leading to the signaling of the cellular interior and activation of the alveolar macrophages (28-31). This leads to a pro-inflammatory cascade defined by the production of specific pro-inflammatory cytokines, such as tumor necrosis factor alpha, followed by induction of interleukin-1 and interleukin-6 (32, 33), recruitment of neutrophils to the wound and a rapid neutrophil infiltration into the lung tissue and airspace (34, 35). In addition to macrophages and neutrophils, other studies suggested that airway epithelial cells, including Clara cells and alveolar type II cells (36-39), are capable of producing a variety of pro-inflammatory cytokines that participate in the innate immune responses.

Several studies used rodent models to investigatethe molecular mechanisms of inflammation-induced increased risk of lung cancer. For instance, overexpression of oncogenic *K-ras* has been shown to lead to the activation of the transcription factor, NF-κB, the enhancement of inflammatory responses and the development of lung adenocarcinomas (21). Another study showed that loss of the lung specific tumor suppressor gene, the G-protein–coupled receptor family C type 5a gene (*Gprc5a*), in mouse lung epithelial cells led to NF-κB activation, followed by aberrant cytokine and chemokine expression in the mouse lung, leading to a chronic inflammatory response, lung epithelial cell proliferation and lung adenocarcinomas (23). Several other studies showed evidence linking carcinogen-induced activation of NF-κB with inflammation and an increased lung cancer risk (18, 20, 22, 24).

Other studies suggested that oxygen and nitrogen species produced during inflammatory responses can cause damage to DNA repair enzymes as well as damage and mutations to cellular DNA (40-42). Some of these mutations may be initiating events in lung tumorigenesis. For instance, oxygen free radicals and other oxidative agents have been shown to cause activating *ras* mutations consisting mostly of G to T transversions (43,44). Four of the *K-ras* mutations found in tumors from mice treated with LPS+NNK in our study consisted of G to T transversions, compared with none in the NNK-treated group. However, additional studies involving a larger number of mice are needed to demonstrate whether oxidative DNA damage may contribute to the *K-ras* mutations in our study. Furthermore, in our experiments, mice were exposed to LPS during the 16 weeks while NNK was administered only during the first 4 weeks. Therefore, it is not clear whether the increased lung tumor incidence was due to an LPS-mediated pulmonary inflammation during any specific period between NNK treatment during the first 4 weeks and tumor appearance at week 17.

Acknowledgements

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