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Tobacco Smoke Promotes Lung Tumorigenesis by Triggering IKKβ- and JNK1-Dependent Inflammation

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SUMMARY

Chronic exposure to tobacco smoke, which contains over 60 tumor-initiating carcinogens, is the major risk factor for development of lung cancer, accounting for a large portion of cancer-related deaths worldwide. It is well established that tobacco smoke is a tumor initiator, but we asked whether it also acts as a tumor promoter once malignant initiation, such as caused by *K-ras* activation, has taken place. Here we demonstrate that repetitive exposure to tobacco smoke promotes tumor development both in carcinogen-treated mice and in transgenic mice undergoing sporadic *K-ras* activation in lung epithelial cells. Tumor promotion is due to induction of inflammation that results in enhanced pneumocyte proliferation and is abrogated by IKKβ ablation in myeloid cells or inactivation of JNK1.

INTRODUCTION

Currently, lung cancer is the leading cause of cancer-related mortalities in men and women, and despite extensive anti-smoking campaigns it still accounts for 15% of all new cancers and 29% of all cancer deaths in the U.S. (Jemal et al., 2008). Among lung cancers, pulmonary adenocarcinoma is the predominant histological type (Jemal et al., 2008; Toh, 2009). Tobacco smoking is the major risk factor, estimated to cause 87% of lung cancer cases in the U.S. (Hecht, 2002). Tobacco smoke (TS) contains ~4000 chemical agents, including over 60 carcinogens: polycyclic aromatic hydrocarbons and N-nitrosamines, such as NNK [4-(methylnitrosamino)-1-(3-pyridyl)-butanone] and aromatic amines (Hecht, 2002). Conversion of these compounds to reactive forms (metabolic activation) results in formation of DNA adducts that cause many of the genetic changes underlying lung cancer. Among these changes, *K-ras*-activating mutations are early events in the pathway leading to lung adenocarcinoma (Herbst et al., 2008). *K-ras* mutations occur in 30%–40% of lung adenocarcinomas, but are infrequent in other lung tumor types or in lung tumors from non-smokers (Berns, 2001). TS also induces pulmonary inflammation (Vlahos et al., 2006), which is believed to play a role in progressive lung destruction in chronic obstructive pulmonary disease (COPD) (Barnes, 2008). Although chronic inflammation was suggested to contribute to tumor initiation through the production of reactive oxygen and nitrogen species that contribute to DNA damage and induction of oncogenic mutations (Hussain et al., 2003), the major effect of inflammation on tumor induction in experimental animals is exerted at the level of tumor promotion (Karin and Greten, 2005). This tumor-promoting effect of inflammation, however, was so far mainly demonstrated in cancers that arise in the context of underlying infections or chronic idiotypic inflammation (Coussens and Werb, 2002; Karin et al., 2006) and its role in tumorigenesis induced by TS or other environmental irritants has not been critically evaluated. Although COPD is known to

SIGNIFICANCE

Tobacco smoking accounts for the majority of lung cancer-related deaths. Despite identification of numerous tumor-initiating carcinogens, it was not established whether tobacco smoke is also a tumor promoter for initiated lung epithelial cells. We now describe two murine models in which tobacco smoke exposure has a well-defined tumor-promoting effect on both chemically and genetically initiated lung cancers. Induction of low-grade inflammation is likely to be an important contributor to the tumor-promoting activity of tobacco smoke, as it depends on IKKβ activity in myeloid cells. These results provide new models and mechanistic insights for understanding tumor induction by a major human carcinogen and demonstrate that the tumorigenic activity of an environmental irritant depends on inflammation.

increase lung cancer risk (Tockman et al., 1987), *K-ras* activation in bronchial epithelial cells can cause an inflammatory response (Ji et al., 2006), and autocrine production of the chemokine CXCL-8 (IL-8) stimulates the growth of *K-ras*-transformed lung adenocarcinoma cells (Karin, 2005; Sparmann and Bar-Sagi, 2004), the role of inflammation in smoking-induced lung tumorigenesis remains to be investigated using appropriate in vivo models. However, in none of the mouse models where TS or compounds derived from it can induce lung cancer was TS shown to act on a different step in the tumorigenic process other than initiation (i.e., induction of oncogenic mutations).

Indeed, previous attempts to ascribe tumor-promoting activity to TS in mice have failed (Witschi, 2005). In fact, mice that were first treated with a carcinogen and subsequently exposed to TS for 5 months showed a significant inhibition of tumor development rather than enhancement (Witschi et al., 1997). After a 4 month recovery phase, TS-exposed mice exhibited the same tumor multiplicity as mice treated with carcinogen alone (Witschi et al., 1997). In NNK-treated mice, TS exposure also failed to increase lung tumor multiplicity (Finch et al., 1996). It has been recognized that in these murine models of TS exposure lung tumorigenesis is influenced by smoke-induced toxicity, manifested by weight loss, thereby necessitating the inclusion of a recovery period (Witschi, 2005). It was also noted that long-term exposure desensitizes mice to TS, as maximal induction of pulmonary inflammation and cell proliferation were observed 1 to 3 weeks after initiation of TS exposure (Witschi et al., 1997). Furthermore, TS exposure can inhibit the metabolic activation of NNK and reduces the formation of NNK-induced O^6-methylguanine DNA adducts (Brown et al., 1999), which strongly correlate with lung tumor yield in A/J mice (Peterson and Hecht, 1991). Taking all of these limitations into consideration, we have devised two murine models in which intermittent, yet prolonged, exposure to mainstream TS (MTS) can successfully promote lung tumor development. We have used these models to determine how the tumor-promoting effect of MTS exposure is accomplished.

RESULTS

To better understand how exposure to TS, which contains many tumor initiators as well as irritants, promotes development of lung cancer, we sought to develop mouse models in which MTS exposure acts as a tumor promoter in addition to its established tumor initiator activity. In the first model, we used NNK as a tumor initiator and MTS as a tumor promoter. First, we optimized the initiating dose of NNK in A/J mice and found it to be 50–70 mg/kg (see Figure S1A, available online). Next, 1 week after intraperitoneal (i.p.) NNK injection into 7-week-old A/J mice, we exposed one group of mice to MTS generated by burning of 4 cigarettes/day, 5 days per week for 1 month, followed by a 1 month rest interval, and repeated this regimen three times. A second group was exposed to 2 cigarettes/day, 5 days per week for 5 months, whereas the third group received filtered air for the same duration, as a control (Figure 1A). All mice were given a final recovery period of 4 months. Whereas MTS-exposed mice failed to gain weight during the exposure period, mice exposed to 4 cigarettes/day for 1 month rapidly resumed weight gain and caught up with air-exposed mice during the 1 month rest interval (Figure 1B). Yet, mice that were continuously exposed to 2 cigarettes/day showed a sustained reduction in body weight.

We analyzed lung tumor multiplicity and incidence by microscopic examination after serial sectioning of lungs at 350 μm intervals as described previously (Curtin et al., 2004). Mice administered 70 mg/kg NNK and exposed to 4 cigarettes/day exhibited significantly increased NNK-initiated lung tumor multiplicity, as well as increased tumor incidence (Figures 1C and 1D). Exposure to 2 cigarettes/day MTS also increased tumor multiplicity and the effect was most pronounced when combined with the highest NNK dose that was tested—70 mg/kg (Figure S1A). Alveolar adenoma was the most common histological type in both air- and MTS-exposed mice, seen in about 70% of each cohort. About 30% of the tumors in both air- and MTS-exposed mice showed features of well-differentiated human papillary adenocarcinoma, including nuclear enlargement, prominent nucleoli, increased mitotic rate, and bronchial invasion (Figure 1E and Figure S1B).

Because the most common mutation found in murine lung tumors induced by NNK is a GGT → GAT transition in codon 12 of the *K-ras* gene (Hecht, 1998), we developed a second model in which we investigated whether intermittent MTS exposure could promote *K-ras*-driven lung tumorigenesis using *K-ras*$^{LA2+/-}$ (*K-ras*^{LA2}) mice, which develop lung cancer in response to sporadic activation of an oncogenic *K-ras*^{asp12} allele through a spontaneous homologous recombination event (Johnson et al., 2001). Six- to eight-week-old, sex-matched *K-ras*^{LA2} mice were exposed to MTS at 4 cigarettes/day for 2–3 weeks followed by 2 week rest intervals on air alone, three times, and lung tumors were analyzed at 5 months of age (Figure 2A). In contrast to A/J mice, weight reduction in *K-ras*^{LA2} mice, which are in the C57BL6 background, was more modest and occurred only during the initial two cycles of MTS exposure (Figure 2B). As found in NNK-initiated mice, MTS exposure increased tumor multiplicity and maximal tumor sizes (Figures 2C and 2D). No tumors were found in MTS-exposed wild-type (WT) mice (Figure 2C), indicating that increased tumor number in MTS-exposed *K-ras*^{LA2} mice is not due to MTS-induced tumor initiation. Similar effects were observed in male and female mice (Figures S2A and S2B). *K-ras*^{LA2} mice also develop thymic lymphoma due to *K-ras*^{asp12} activation in thymocytes (Johnson et al., 2001). No statistically significant differences in the incidence of thymic lymphoma were detected between air- and MTS-exposed *K-ras*^{LA2} mice (Figure S2C), suggesting that the tumor-promoting effect of MTS is limited to the primary site of exposure—the lung. Most pulmonary tumors in air- or MTS-exposed *K-ras*^{LA2} mice were alveolar adenomas (Figure 2E and Figure S2D), corresponding to a relatively early stage of lung cancer development, as reported previously (Johnson et al., 2001). Interestingly, tumors in MTS-exposed mice were more vascularized than those in air-exposed controls (Figures S2E and S2F). Thus, MTS exposure promotes development of both chemically and genetically induced lung cancer and this constitutes strong evidence that TS acts as a tumor promoter and not only as an initiator.

We investigated whether the tumor-promoting effect of MTS exposure is due to induction of inflammation. We examined the subacute inflammatory pulmonary response of C57BL6 male