Carcinogenesis vol.26 no.9 pp.1488–1492, 2005
doi:10.1093/carcin/bgi148
Advance Access publication June 1, 2005

COMMENTARY

Carcinogenicity studies of inhaled cigarette smoke in laboratory animals: old and new

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A new study demonstrates that lifetime whole-body exposure of B6C3F$_1$ mice to high doses of cigarette smoke robustly increases lung cancer incidence compared with sham exposed animals. This is the first study to demonstrate a strong effect of inhaled cigarette smoke on lung cancer in an animal model. This commentary attempts to put the new results in perspective with the existing literature on cigarette smoke inhalation studies in animals and discusses strengths, limitations and possible applications of available models.

Introduction

The first study to show a robust increase in lung cancer in an animal model of cigarette smoke inhalation appears in this issue of *Carcinogenesis* (1), more than 50 years after the initial epidemiologic studies linking smoking and lung cancer in humans (2,3). Why has this been so difficult to achieve? One reason is that humans actively and religiously inhale cigarette smoke to satisfy their extraordinary craving for nicotine, while animals are affronted by this toxic mixture and will do what they can to avoid it. This commentary will attempt to put the results of the new study in perspective with other inhalation studies of cigarette smoke, discuss the rationale for animal models of cigarette smoke exposure, the strengths and limitations of currently available models, and the need for integration of carcinogen biomarker data in future studies.

Previous cigarette smoke inhalation studies

Early studies were summarized by Wynder and Hoffmann (4). Subsequently, comprehensive reviews have been published by the International Agency for Research on Cancer (5,6), Coggins (7–9) and Witschi (10). These reviews present many details of published work, and no attempt will be made to repeat that here. Representative studies will be discussed and important conclusions highlighted. Experiments have been carried out in hamsters, rats, mice, dogs, rabbits, non-human primates and ferrets.

Hamster

Consistently, pronounced alterations of the larynx including carcinoma were induced by exposure of Syrian golden hamsters to cigarette smoke. In a study carried out by Dontenwill *et al*. (11) involving 4440 hamsters, exposed nose only to the smoke of various cigarettes, the severity of alterations in the larynx depended on smoke dose and duration of treatment. No such alterations were observed in sham exposed animals or in animals exposed only to the gas phase of smoke (separated from particulates by passage through a standard glass fiber filter). Other experiments by the Dontenwill group as well as extensive investigations by Bernfeld, Homburger and others using strain BIO® inbred hamsters produced similar results [reviewed by the International Agency for Research on Cancer (6)]. Dontenwill *et al*. (11) also demonstrated that cigarette smoke acted as a tumor promoter for induction of larynx tumors in animals treated with a single dose of 7,12-dimethylbenz[*a*]anthracene, which itself did not induce tumors of the larynx. Similar results were obtained by Hoffmann *et al*. (6). The large Dontenwill study also tested various types of modified cigarettes, including ones with different types of filters or tobacco filler (11). All modified cigarettes produced lower incidences of laryngeal tumors than did the reference cigarette.

The estimated concentration of smoke particles in the larynx was ~300 times greater than that in the lungs and bronchi under the conditions of these experiments (11). This would explain why tumors were observed in the larynx rather than the lung.

The results of the hamster studies were generally consistent with those obtained upon application of cigarette smoke condensate to mouse skin. The mouse skin studies unquestionably demonstrate that non-volatile constituents of cigarette smoke have tumor initiating, tumor promoting and complete carcinogenic activity (12). This cannot be due to gas phase constituents because these are lost during preparation of the condensate. Mouse skin studies also showed decreased tumorigenicity resulting from application of condensates prepared from cigarettes modified in ways similar to those in the hamster inhalation experiments (12).

Rat

Mauderly *et al*. (13) recently demonstrated convincing, although moderate, increases in tumors of the lung and nasal mucosa in rats exposed to cigarette smoke. Male and female F344 rats (n = 81–178/gender) were exposed whole body 6 h per day, 5 days per week for up to 30 months to smoke from 1R3 research cigarettes or to clean air. Cigarette smoke exposure significantly increased the incidence of non-neoplastic and neoplastic proliferative lung lesions in females. Non-significant increases were observed in males. The combined incidence of bronchioalveolar adenomas and carcinomas was 14% in the high exposure (250 mg/m^3 particulate) group, 6% in the low exposure (100 mg/m^3) group and zero in controls. Mutations in codon 12 of the K-*ras* gene occurred in 4 of 23 tumors; 3 were G–A transitions and one a G–T transversion. Both males and females had significant increases of nasal cavity neoplasia (13).

Abbreviations: B[*a*]P, benzo[*a*]pyrene; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PREP, potential reduced exposure product.

The results of the Mauderly study (13) stand in contrast to those of previous investigations (6,7) of cigarette smoke exposure in rats, which did not consistently demonstrate significant increases in tumors of the lung, nasal cavity or any other site. The major difference between the Mauderly study and previous ones was dose. Rats exposed whole body to radiolabeled cigarette smoke had over twice the amount of radioactivity in their lungs than rats exposed nose only. The weekly exposure times in the Mauderly study were also longer than in nose only studies. Grooming of contaminated pelt may also have contributed to the induction of lung and nasal cavity tumors, perhaps through ingestion of tobacco-specific nitrosamines, which induce tumors of this type when given orally (14).

Mouse

In the study reported by Hutt *et al*. in this issue (1), female $B6C3F_1$ mice were exposed whole body, 6 h per day, 5 days per week, for 920–930 days to mainstream cigarette smoke (250 mg/m^3), or sham exposed. Significantly elevated incidences of lung adenoma (28% in treated and 6.7% in control), total benign pulmonary neoplasms (31 versus 7%), adenocarcinoma (20 versus 2.8%) and distal metastases (1.5 versus 0.3%) were observed in the cigarette smoke exposed mice. These findings are even more remarkable because they were obtained in a strain of mouse which has a low baseline incidence of pulmonary neoplasia (15).

An A/J mouse model that is responsive to cigarette smoke has been described by Witschi *et al*. (10). Benign lung tumors were induced in this highly susceptible strain by exposure to a mixture of 89% cigarette sidestream smoke and 11% mainstream smoke. The animals were exposed for 5 months, then allowed a 4 month recovery period. In 18 individual studies reported by four different laboratories, a significant increase in lung tumor multiplicity was observed in 15 studies and a significant increase in lung tumor incidence in 10. The increases in tumor multiplicity were generally small, from ~1 tumor per mouse to ~2.8, following exposure to 50–170 mg/m^3 of total suspended particulates. The increase in tumor multiplicity observed in this model was due to a component of the gas phase of tobacco smoke. This was consistent with early studies by the Leuchtenbergs, but contrasted with the hamster data described above (6). An advantage of this model is that it provides a relatively simple and inexpensive method to induce lung tumors with cigarette smoke. On the other hand, there are some features which require further investigation. The animals treated with smoke do not gain weight as quickly as those treated with filtered air, which complicates interpretation of the data. The 4-month recovery period is absolutely necessary for observation of increased lung tumor multiplicity, but the reason for this is not clear. Nevertheless, this model has already been applied fairly widely, particularly in chemoprevention studies (10).

Other species

Smoke inhalation studies have been carried out with dogs trained to inhale cigarette smoke through tracheostomata and by nasal inhalation (6,8). None of these studies provided convincing evidence of pulmonary tumor induction. Some studies have also been performed with rabbits and small numbers of non-human primates, all with negative results (6,8). The ferret has been suggested as a useful model for inhalation toxicology because of the ease with which measurements can be made and the resemblance of its airways to those of humans (16). Although no carcinogenicity studies of cigarette smoke alone have been reported in ferrets, there have been a number of recent investigations which have examined the effects of β-carotene or lycopene supplementation on cigarette smoke-induced changes (17–22).

Why do we need an animal model?

There is overwhelming evidence that cigarette smoking is the major cause of lung cancer in the world. Smoking is also a cause of cancers of the larynx, pharynx, nasal cavity, esophagus, stomach, liver, pancreas, bladder, kidney, ureter, cervix and of myeloid leukemia (23). Overall, cigarette smoking causes 30% of all cancer death in developed countries (24). Given these well-established facts, confirmed repeatedly in epidemiologic studies, why is it necessary to have an animal model of cigarette smoke-induced cancer? When some of the animal experiments described above were carried out, the epidemiology was still evolving, and the prominent position of cigarette smoking as a major cause of cancer in humans was not fully appreciated. It was important to demonstrate that cigarette smoke could in fact cause cancer in animal models, in order to bolster the epidemiologic evidence. Today, there is no argument about cigarette smoking and lung cancer, even from the tobacco industry. Nevertheless, animal models of cigarette smoke-induced cancer are still important for several reasons.

First, there are new cigarette brands appearing on the market which make direct or implied claims of lower toxicity and carcinogenicity. These products have been called 'potential reduced exposure products' or PREPs (25). Objective evaluation of the health effects of PREPs is critical. Approaches to this problem have focused to date on properly designed clinical studies of smokers which incorporate appropriate biomarkers related to tobacco-induced disease (26,27). An animal model of cigarette smoke-induced cancer would certainly be an important addition to the evaluation process. But it would be necessary to use realistic cigarette smoking conditions in such a model. The hamster inhalation experiments described above used the standard Federal Trade Commission conditions (puff volume, 35 ml; puff duration, 2 s; puff frequency, 1 per min) to test a variety of modified cigarettes: all produced fewer alterations in the larynx than did a standard blend (11). These modified cigarettes incorporated some of the technology used in light and ultra-light cigarettes that have been on the market for many years (28). The widespread use of these cigarettes has not resulted in decreased lung cancer mortality, and epidemiologic data show that the risk for lung cancer is no different in smokers of these cigarettes compared with conventional medium tar filtered brands (29,30). Furthermore, there is no difference in the uptake of known carcinogens in smokers of these cigarettes versus conventional brands (31). It is now clear that people do not smoke light cigarettes in the same way machines do, and that individual smoking topography can vary widely (32). It will be necessary to take this into account when designing animal model experiments to evaluate PREPs.

Second, an animal model of cigarette smoke-induced lung cancer could be critical for the evaluation of chemopreventive agents. Chemoprevention is an important strategy for protection of ex-smokers and addicted current smokers, both at high risk for lung cancer. Unfortunately, there are presently no chemopreventive agents which are effective against lung

cancer in clinical trials (33). There are many promising agents which have shown efficacy in animal models of lung cancer which use cigarette smoke carcinogens such as benzo[*a*]pyrene, (B[*a*]P) or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, (NNK) (34). Efficacy in an animal model of smoke inhalation could support the further development of these agents for clinical trials in smokers. Limited research in this area has been performed in the A/J mouse model, with modest results to date (10). Proper pre-clinical evaluation of chemopreventive agents in animal models is critical and can help to avoid negative results in costly clinical trials.

Third, animal models can potentially provide insights on mechanisms of cigarette smoke-induced cancer. An understanding of mechanisms is important for the rational development of both preventive and therapeutic approaches, by identifying important targets. While we have achieved vast mechanistic understanding of tobacco smoke carcinogenesis, there are still many features of the process which are unclear (35,36). We can address these issues using individual tobacco carcinogens or the whole mixture. The latter approach is more realistic, but also far more difficult. Nevertheless, the results of such studies using an appropriate smoke inhalation model could be very revealing. In one set of studies, Gupta *et al*. have investigated DNA adducts in tissues of rodents exposed to cigarette smoke (37). Their results indicate that the major adducts detected by ^{32}P-postlabelling do not result from exposure to polycyclic aromatic hydrocarbons, widely considered to be important in cigarette smoke-induced cancer, but rather to increased levels of endogenous DNA adducts.

Strengths and limitations of smoke inhalation models

There are some problems with cigarette smoke inhalation models which appear to be universal (5). Rodents are obligatory nose breathers with intricate and highly developed nasal turbinates different from those of humans. This leads to different deposition patterns in rodents and humans which can hardly be avoided. Animals which are being forcibly exposed to cigarette smoke change their breathing patterns and undergo avoidance reactions. Their shallow breathing patterns are quite different from the active inhalation of cigarette smoke by humans. The continual exposure of rodents to high doses of cigarette smoke results in body weight decreases which can complicate the interpretation of data. There is no perfect exposure system for laboratory animals. Nose only systems require extensive handling and restraint of the animals which can induce stress while whole-body exposures result in deposition of particles on the pelt and oral exposure via grooming. Experiments with larger animals that have been trained to inhale smoke using various strategies have been generally unsuccessful, are prohibitively expensive, and probably could never be done in the current regulated era. Nevertheless, if one remains cognizant of these difficulties, an effective smoke inhalation model could still be very useful for the reasons discussed above. There are general limitations of all animal models of cancer, both conventional and genetic, but few would question the importance of these models in studies of cancer prevention and therapy.

The model described by Hutt *et al*. (1) has the clear advantage of strong induction of lung cancer by cigarette smoke, with the incidence of adenocarcinoma being 10 times greater in smoke-exposed than in sham-exposed mice. Pulmonary adenocarcinoma, as induced in these mice, is now the most common histologic type of lung cancer in humans in the USA (38). The tumors induced in mice in this study appear to have many histopathological features similar to those seen in smokers. Furthermore, the presence of K-*ras* mutations and epigenetic silencing of the DAP-kinase and RAR-β genes have parallels in human lung cancer. A limitation of this model is the long exposure period required (920–930 days). Few institutions would have the physical facilities and other resources required to carry out such a study. Thus, in spite of its obvious attractive features, it seems unlikely that this model will be widely used.

The Witschi A/J mouse lung tumor model is already being used in a number of different laboratories (10). Its major advantage is that it provides a relatively inexpensive way to induce lung tumors with cigarette smoke. The methodology has been described in detail and is applicable in small spaces with relatively limited equipment (39). Multiple published studies support the simplicity of this system. The major limitation is that only a small increase in benign lung tumors is observed in a highly susceptible mouse strain. This pulmonary response is not specific to cigarette smoke. The lung is the major target tissue in the A/J mouse, independent of the carcinogenic agent. Many agents which are not considered lung carcinogens induce lung tumors in this strain (40). Furthermore, the lack of weight gain observed during treatment and the necessity for a recovery period can complicate interpretation of results in this assay.

The hamster inhalation model is the only other one in which tumor induction by cigarette smoke has been reproducibly achieved. Strengths of this model include the large studies that have been carried out in different laboratories with similar results, and the consistency of the data with mouse skin painting studies, for which there is also a vast data base. Limitations include the lack of tumor induction in the lung and the considerable expense of the assay which requires placement and removal of hamsters from tubes. Treatment related weight loss is another problem. It does not appear that this model is being used at the present time.

While the tumor response in each model is due to exposure to the whole mixture, there are clearly differences between the models in the responsible components of the mixture. These differences need to be kept in mind when interpreting data from these experiments. The lung tumors induced in A/J mice are due to a gas phase constituent of cigarette smoke, perhaps 1,3-butadiene or ethylene oxide. If a chemopreventive agent is inactive in this system, it can reasonably be concluded that the agent does not inhibit tumor induction by a volatile constituent of smoke, but no conclusion could be made with regard to particulates. On the other hand, if a chemopreventive agent were inactive in the hamster model, the opposite conclusion could be reached. It is not clear at present whether gas phase or particulate constituents are responsible for the tumors observed in B6C3F$_1$ mice.

Calibration of dose and the use of carcinogen biomarkers

The dose used in the Hutt *et al*. study, 250 mg/m^3 particulates, was estimated by the authors to correspond to a human smoking 3–4 packs of cigarettes per day, a level that is reached in few smokers (1). If this is correct, the daily dose of the representative cigarette smoke lung carcinogens B[*a*]P and NNK would be ~0.5–0.7 and 9–12 μg per day, respectively, or total doses of 460–640 μg B[*a*]P and 8300–11 000 μg NNK

in the 920-day experiment (41). It is doubtful that these amounts of the pure compounds could induce lung tumors in B6C3F1 mice. Of course, these calculations can be grossly inaccurate for a number of reasons. A much more useful way to measure carcinogen dose in inhalation experiments would be to assess biomarkers of exposure. Since the mid-1980s, a huge literature on measurement of biomarkers in smokers has evolved (42). This includes studies on urinary metabolites, hemoglobin adducts and DNA adducts. 1-Hydroxypyrene is a well-established urinary biomarker of polycyclic aromatic hydrocarbon uptake while the NNK metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol is an established biomarker of NNK uptake. Urinary metabolites of other cigarette smoke constituents such as benzene and 1,3-butadiene have also been used as biomarkers of carcinogen uptake in smokers (43). Hemoglobin adducts of aromatic amines, ethylene oxide and other cigarette smoke constituents have been widely measured in humans (42). Quantitation of DNA adducts is another approach to determine dosimetry of tobacco smoke carcinogens (44). This has been accomplished in some cases using specific methods such as mass-spectrometry or HPLC-fluorescence, but much more commonly (and perhaps less accurately) by immunoassay and ^{32}P-postlabelling. Unfortunately, carcinogen biomarkers have seldom if ever been applied in published animal carcinogenicity studies of inhaled cigarette smoke. Some studies have used tracers to determine cigarette smoke dose to the lung, many have measured carboxyhemoglobin, and a few (5–9) have reported nicotine or cotinine levels in the lung. The lack of quantitation of carcinogen biomarkers is an important omission which should be corrected in future studies, as it would facilitate a comparison between carcinogen uptake in exposed animals and smokers. Such a comparison is critical for the interpretation of data from smoke inhalation studies.

Conclusion

The robust induction of pulmonary adenocarcinoma and other changes by exposure of mice to cigarette smoke represents a considerable step forward in the study of tobacco-induced lung cancer. It is the first example of a strong carcinogenic response in the lungs of animals exposed to cigarette smoke. The new model provides a test system in which questions pertinent to evaluation of new tobacco products, development of chemopreventive agents, and mechanisms of carcinogenesis can be addressed. There are still drawbacks, some of which are common to all smoke inhalation models. While studies in smokers, who are available by the millions, are the preferred way of investigating mechanisms and prevention of tobacco-induced cancer, animal models have their place and can contribute to the development of methods to prevent and treat cancers caused by tobacco use.

Acknowledgement

Research on tobacco and cancer in the author laboratory is supported by grants CA-81301 and DA-13333 from the NIH and RP-00-138 from the American Cancer Society.

Conflict of Interest Statement: None declared.

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Received May 17, 2005; accepted May 29, 2005

Note added in proof

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