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Towards the validation of a lung tumorigenesis model with mainstream cigarette smoke inhalation using the A/J mouse

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ABSTRACT

A generally accepted and validated laboratory model for smoking-associated pulmonary tumorigenesis would be useful for both basic and applied research applications, such as the development of early diagnostic endpoints or the evaluation of modified risk tobacco products, respectively. The A/J mouse is susceptible for developing both spontaneous and induced lung adenomas and adenocarcinomas, and increased lung tumor multiplicities were also observed in previous cigarette smoke inhalation studies. The present study was designed to collect data useful towards the validation of an 18-month mainstream smoke (MS) inhalation model. Male and female A/J mice were exposed whole-body at three MS concentration levels for 6 h/day, and the results were compared to a previous study in the same laboratory and with a similar design. A linear MS concentration-dependent increase in lung tumorigenesis was observed with similar slopes for both sexes and both studies and a maximal 5-fold increase in multiplicity beyond sham control. The minimal detectable difference in lung tumor multiplicity for the current study was 37%. In the larynx, papillomas were detectable in all MS-exposed groups in a non-concentration dependent manner. No other extra-pulmonary MS-dependent neoplastic lesions were found. Gene expression signatures of lung tumor tissues allowed a clear differentiation of sham- and high dose MS-exposed mice. In combination with data from previous smoke inhalation studies with A/J mice, the current data suggest that this model for MS inhalation-induced pulmonary tumorigenesis is reliable and relevant, two crucial requirements towards validation of such a model.

1. Introduction

Cigarette smoking is a cause of severe diseases, including lung cancer (International Agency for Research on Cancer, 2004). While more and more information is becoming available on the pathogenesis of smoking-related lung cancer (US Department of Health and Human Services, 2010), a comprehensive understanding of the actual causative agents in smoke and the mechanisms involved is still missing. To some extent, this knowledge gap is related to the lack of a generally accepted laboratory animal model for mainstream smoke (MS) inhalation-inducible lung cancer. Such a model, once established, could be used for etiological and mechanistic research, for research on diagnostic and therapeutic means, and for the evaluation of modified risk tobacco products. Such models have recently been called for by the US FDA (US Food and Drug Administration Center for Tobacco Products, 2012) and the US IOM (Institute of Medicine, 2012), in particular for comparative assessments.

The purpose of bioassays on carcinogenesis is to identify carcinogenic properties of test materials in laboratory rodents in order to evaluate a carcinogenic potential for humans (Organisation for Economic Co-operation and Development, 1981, 2009). In line with regulatory guidance for conducting bioassays on carcinogenesis, laboratory rats and mice are most commonly exposed for an appreciable portion of their lifespan. In the case of smoking, a carcinogenic potential has already been established in humans, and

Abbreviations: ANOVA, analysis of variance; ETSS, environmental tobacco smoke surrogate; MDD, minimal detectable difference; MS, mainstream smoke; MS-75, MS-150, MS-300, groups exposed to MS concentrations of 75, 150 and 300 mg/m^3 TPM, respectively; R^2, coefficient of determination; RLE, relative log expression; SD, standard deviation; SE, standard error of the mean; TPM, total particulate matter.

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bioassays are required to model the human disease pathogenesis to the extent possible for the above-mentioned applications. In terms of lung cancer, laboratory rodents mainly develop peripheral pulmonary adenomas that may progress to adenocarcinomas, while humans may develop various histological types of highly invasive bronchial and bronchiolar-alveolar carcinomas (Schleef et al., 2006) with an increasing fraction of adenocarcinomas over the last decades (Devesa et al., 2005).

Despite many years of research, no model for MS-induced lung tumorigenesis could be established that is generally accepted (Coggins, 2010). However, there are three rather recent developments, which may eventually qualify. (1) Lifetime MS inhalation studies have been recently reported on F344 rats and B6C3F1 mice, in which statistically significant increases in lung tumors were found in females (Hutt et al., 2005; Mauderly et al., 2004). However, the response for male rats was negative, and male mice were not tested. Furthermore, it seems that these studies have not been repeated anywhere to test for reproducibility. (2) A relatively pronounced increase in lung tumorigenicity in male and female Swiss mice was obtained when MS inhalation exposure was started immediately after birth (Balansky et al., 2007). These results seem to be reproducible in the same laboratory (Balansky et al., 2009), but apparently this study design has not been reproduced in other laboratories. Furthermore, the biological relevance of this particular exposure scenario for the assessment of smoking-related tumorigenesis remains to be established. (3) A series of positive lung tumorigenesis inhalation studies have been conducted using whole-body exposure of A/J mice to an environmental tobacco smoke surrogate (ETSS) (Stinn et al., 2005; Witschi, 2005). In these studies, mice were exposed for 5 months followed by a 4-month post-inhalation period (5 + 4-month schedule), which was needed for the smoke-induced tumors to develop beyond incidences found in sham-exposed controls. Using the same exposure schedule, studies on MS inhalation were also negative at the end of the 5-month inhalation period but positive at the end of the 4-month post-inhalation period (Curtin et al., 2004; Stinn et al., 2010).

In an 18-month study with A/J mice, the need for a post-inhalation period was confirmed for 5- and 10-month MS inhalation periods, but MS inhalation for 18 months was sufficient to elicit a concentration-dependent lung tumor response without the need for a further post-inhalation period (Stinn et al., 2012). The susceptibility of the A/J mouse to the development of spontaneous and chemically induced lung adenomas and adenocarcinomas seems to be related to a propensity of the K*ras* proto-oncogene for mutation and increased transcription (Chen et al., 1994; To et al., 2006). Mutated K*ras* genes have frequently been found in human lung adenocarcinomas of smokers (Porta et al., 2009). In view of the above, this model warrants further investigation of its reliability and biological relevance, two crucial requirements of toxicological method validation (e.g., Interagency Coordinating Committee on the Validation of Alternative Methods, 1997).

With the aim of generating data towards validating the A/J mouse model, the objectives of the present study were

- to generate data on intra-laboratory reproducibility of the lung tumor response in A/J mice exposed to MS inhalation for 18 months and to discuss inter-laboratory reproducibility based on published shorter-term smoke inhalation studies;
- to extend the information on model characteristics, such as sex dependence, and concentration–response relationship, and extra-pulmonary tissue effects;
- to assess data variability and discriminatory power; and
- to add further mechanistic data for the evaluation of the biological relevance of the model, i.e., the gene expression signature in tumor tissues.

2. Materials and methods

2.1. General study design

Due to the objective of reproducing the data from the previous 18-month inhalation study (designated as Study 1, Stinn et al., 2012), the basic study design and methods were very similar for the current study (Study 2). In order to align as much as possible to regulatory guidance available for the carcinogenicity testing of chemicals (Organisation for Economic Co-operation and Development, 1981, 2009), Study 2 additionally included female mice as the second sex and the histopathological examination of extra-pulmonary organs and tissues. For a better characterization of the MS concentration–response curve, a third concentration was added, which was below the ones previously used, because the high concentration in Study 1 was considered the maximum tolerated MS concentration. Thus, in Study 2, male and female mice were whole-body exposed to 75, 150, and 300 mg/m^3 total particulate matter (TPM) (MS-75, MS-150, and MS-300) for 6 h/day and 5 days/week for 18 months (interim dissection after 10 months). A sham-exposed control group was treated the same way except for MS inhalation. A post-inhalation period of 2 days was added for a satellite group of mice exposed for 18 months that were allocated to the investigation of gene expression patterns in lung tumor tissue. This short post-inhalation period was expected to down-regulate most of the acute MS exposure related induction of gene expression in order to allow a characterization of longer-term effects that may be characteristic for the tumorigenic process.

In Study 1, MS was generated using the standard reference cigarette 2R4F. Due to the diminishing stock of 2R4F cigarettes, 3R4F cigarettes were used in Study 2 for MS generation (University of Kentucky, Lexington, KY) (for specifications see http://www.ca.uky.edu/refcig/). Both reference cigarette types display equivalent MS composition as well as in vitro and in vivo toxicity (Roemer et al., 2012). MS generation was performed in basic accordance with international standards (International Organization for Standardization, 1991, 2000). Analytical characterization of the MS was performed as previously reported (Stinn et al., 2012).

The approval for the performance for both studies was obtained according to the Belgium Law on Animal Protection (Belgian Federal Public Service, 2004). The studies were performed in an AAALAC-accredited facility (Association for the Assessment and Accreditation of Laboratory Animal Care International, 1991), where care and use of the mice were in accordance with the American Association for Laboratory Animal Science (AALAS) Policy on the Humane Care and Use of Laboratory Animals (http://www.aalas.org).

2.2. Test system

Male and female A/J mice bred under specified pathogen-free conditions (The Jackson Laboratory, Bar Harbor, Maine, USA) were obtained through Charles River France (L'Arbresle, France). The age of the mice was between 6 and 10 weeks at arrival and between 8 and 12 weeks at start of the inhalation, as in Study 1.

The health status of six male and six female mice was confirmed serologically (Bioreliance, Rockville, MA), bacteriologically, parasitologically (Harlan, UK), and histopathologically. Eight of 12 mice were positive for *Klebsiella oxytoca*, which was not considered to impact the study quality since there was no pattern of characteristic lesions that might have been associated with Klebsiella infections.

Within 1 week after arrival, the mice were individually identified with subcutaneous transponders (Triple A Trading, Tiel, the Netherlands). After random allocation to groups, the mean body weight per group at the start of exposure was approximately 22 g for the male and 18 g for the female mice, with relative standard deviations (SD) of less than 11%. Twenty and 64 mice per group and sex were allocated for the 10-month interim and 18-month final dissection time points, respectively. The mice were fed irradiated Harlan Teklad 2014 diet at libitum (Harlan, Blackthorn, UK), except during exposure. Filtered tap water was offered during and between exposures. Irradiated softwood granulate bedding material, type Lignocel BK8/15 (Tecnilab, Someren, the Netherlands) was used. The position of the cages in the whole-body exposure chambers was rotated on a weekly basis. There were at least six air changes per hour in the exposure chambers, and the equivalent flow rate through each exposure chamber was at least 80 l/min. The mean temperature and the mean relative humidity in the sham-exposure chambers during exposure was 22.3 ± 0.6 °C (mean ± SD) and 55.7 ± 2.6% (mean ± SD) respectively. These conditions were considered representative of the MS chambers as well. The exposure period started with adaptation periods of 2, 3, 4, and 5 h/day (3 days each) prior to the final 6 h/day. Mice that died during the first 6 weeks of the study were replaced.

2.3. Biological endpoints

In-life observations and determinations, necropsy, organ weights, hematology (without differentiation of leukocytes) and respiratory tract histopathology were performed as previously described (Stinn et al., 2010). All mice that died