Tobacco Smoke–Induced Lung Cancer in Animals—A Challenge to Toxicology (?)

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Tobacco smoke is a known human carcinogen that primarily produces malignant lesions in the respiratory tract, although it also affects multiple other sites. A reliable and practical animal model of tobacco smoke–induced lung cancer would be helpful for in studies of product modification and chemoprevention. Over the years, many attempts to reproduce lung cancer in experimental animals exposed to tobacco smoke have been made, most often with negative or only marginally positive results. In hamsters, malignant lesions have been produced in the larynx, but not in the deeper lung. Female rats and female B6C3F1 mice, when exposed over lifetime to tobacco smoke, develop tumors in the nasal passages and also in the lung. Contrary to what is seen in human lung cancers, most rodent tumors are located peripherally and only about half of them show frank malignant features. Distant metastases are extremely rare. Male and female strain A mice exposed to 5 months to tobacco smoke and then kept for another 4 months in air respond to tobacco smoke with increased lung tumor multiplicities. However, the increase over background levels is comparatively small, making it difficult to detect significant differences when the effects of chemopreventive agents are evaluated. On the other hand, biomarkers of exposure and of effect as well as evaluation of putative carcinogenic mechanisms in rats and mice exposed to tobacco smoke allow detection of early events and their modification by different smoke types or chemopreventive agents. The challenge will be to make such data broadly acceptable and accepted in lieu of having to do more and more long term studies involving larger and larger number of animals.

Keywords A/J Mouse, Bioassays, Lung Tumors, Tobacco Smoke

HISTORICAL PERSPECTIVE

Almost a century ago, at the end of the First World War (1914–1918), it began to be suspected that the smoking of cigarettes was responsible for the steeply increased occurrence of a previously extremely rare disease: lung cancer (Witschi 2001). In 1929, the German physician Fritz Linckit, the doctor “most hated by the tobacco industry” showed in a “case series” that lung cancer patients were likely to be smokers and that in countries where women smoked as much as men, lung cancer rates were about the same in both sexes (Proctor 1999). Shortly thereafter, another physician published a small note entitled “Cigarette smoke as a cause for lung cancer—a suggestion” (Mertens 1930). This seems to have been the first attempt to produce lung cancer with tobacco smoke in experimental animals. Mertens forced tobacco smoke with a rubber bulb into a glass dessicator and mice were exposed daily for up to 15 months. The results seemed to be disappointing. Although evidence of inflammation of the airways and the lung parenchyma were readily visible, neoplastic lesions were only found in two animals. The author doubted that tobacco smoke had been the culprit.

Epidemiological and experimental research on the health effects of tobacco smoking continued in Germany and elsewhere. By the early 1950s, epidemiological investigations in England and in the USA had provided strong evidence for an association between the smoking of cigarettes and lung cancer. By now it is well documented that smoking is the cause of the overwhelming majority of cases (Doll 1998; IARC 2004). Animal experiments were also initiated more than 50 years ago. As early as in 1952, tobacco smoke had been found to cause lung cancer in strain A mice, but the experiments were not conclusive, being positive in one experiment and negative in another (Essenberg 1952, 1957; Essenbarg et al. 1955). In 1961 it was reported that the painting of tobacco smoke condensate (“tar”) onto the back of mice did produce benign and malignant skin tumors. This observation lead to the eventually generally accepted conclusion that the main carcinogen in cigarette smoke was benzo(a)pyrene, although at the time not all investigators were unequivocally in favor of this hypothesis (Wynder 1961; Druckrey 1961).

In the 1970s, several major attempts were made to show that inhalation of tobacco smoke would produce lung cancer in mice, rats, hamsters, dogs, and even monkeys. Although some of these experiments involved thousands of animals, the aggregated evidence failed to confirm the human epidemiological studies. Inhalation of tobacco smoke, even if conducted over the lifetime of animals, failed to reproduce the disease seen in man (Coggins 2002, 2007). Eventually, efforts to develop whole

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animal models of tobacco smoke carcinogenesis were abandoned. Some of this might have been an involuntary consequence of the ill-fated effort of the National Cancer Institute to develop a less harmful cigarette (Kluger 1996) and the Carter administration was not enamored by the idea that taxpayer money should be spent in order to “improve” a consumer product known to cause widespread health problems. On the other hand, “mechanistic” research flourished, most of it conducted with in vitro systems. The mode of action of such tobacco smoke carcinogens as polycyclic aromatic hydrocarbons and tobacco specific nitrates was extensively analyzed. This research culminated in what was called, in an accompanying editorial, “the smoking gun,” the identification of benzo(a)pyrene adducts at p53 mutational hotspots in HeLa and human bronchial epithelial cells (Denissenko et al. 1996).

In the 1990s, several developments led to a renewed interest in whole-animal models of tobacco smoke inhalation: the possibility that active smoking might increase the risk to develop lung cancer in workers exposed to certain industrial carcinogens, such as radionuclides (Finch et al. 1998); the emergence of environmental tobacco smoke as a major public health problem (NCI 1999); the recognition that it would be desirable to develop modified products which might result in less harm to smokers (Wagner et al. 2000); and the emphasis of the concept of chemoprevention (Hong and Sporn 1997). Somehow, history then seemed to repeat itself.

As had been done originally by Essenberg (1952), strain A/J mice were again selected to see whether tobacco would produce an increased incidence and multiplicity of lung tumors. The first two experiments were inconclusive, possibly because the concentration of tobacco smoke was too low (Witschi et al. 1995) or because the animals were killed too soon after cessation of smoke exposure (Finch et al. 1996). However, in 1997 the first of a series of studies reported that exposure of strain A mice for 5 months to a mixture of 89% cigarette sidestream and 11% mainstream smoke, followed by a month recovery period in air, significantly increased lung tumor multiplicity in strain A mice (Witschi et al. 1997a, 1997b). Since then, the finding has been successfully reproduced in several independent studies from different laboratories (D’Agostini et al. 2001; Curtin et al. 2004b; Stinn et al. 2005; Wang et al. 2005). The strain A/J mouse model and the adopted experimental protocol seemed to be a promising tool for studying tobacco smoke carcinogenesis and its eventual modulation through product modification or administration of chemopreventive agents.

THE STRAIN A MOUSE LUNG TUMOR ASSAY

The model has several advantages. as well as some disadvantages, as was discussed recently (Witschi 2005a). Among its many advantages are very good intra- and interlab reproducibility. The number of mice per experimental and control group can usually be kept between 20 and 30, thus not necessitating large numbers of experimental animals. Experiments can be completed within a 1-year period. Inclusion of positive controls—strains A mice injected with urethane—will show that the animals have the required sensitivity to carcinogens. The predictability of the model should encourage the verification of important new or unexpected findings in repeat experiments. Dose-response information is available. A plotting of the available data obtained by exposing strain A mice to tobacco smoke has a slope significantly different from zero. The slope is shallow and indicates that tobacco smoke is a weak lung carcinogen in experimental animals. At one time it was hoped that using transgenic animals rather than the plain A/J strain would increase the sensitivity of the assay. Several mutants were used such as rasH2 Tg mice (Curtin et al. 2004b), strain A mice carrying alterations in K-ras, p53, or Ink4a/Arf (Wang et al. 2005) or carrying a dominant negative p53 mutation (DeFlora et al. 2003b). However, the results from these studies demonstrated that such animals are only marginally more sensitive to tobacco smoke than are the corresponding wild types and may thus not necessarily justify the greatly increased costs.

The model allowed reexamining a problem that first had surfaced almost 40 years ago. In 1971, the Leuchtenbergers (Leuchtenberger and Leuchtenberger 1971) described that the gas phase of tobacco smoke was as efficient in inducing lung tumors in Snell mice as was full tobacco smoke. This finding was in contrast to what had been found in hamsters, where only the particle phase, but not the gas phase, had induced laryngeal tumors (Dontenwill et al. 1973). In both 1997 and 2005 it was again reported that the gas phase of cigarette smoke, obtained by passing full smoke through a HEPA filter, induced as many lung tumors as did the full smoke mixture (Witschi et al. 1997a; Witschi 2005b). On the other hand, a more recent study, also done in strain A mice but using a different technique to generate the gas phase, failed to associate tumor development with the gas phase (Stinn et al. 2006).

These different observations deserve attention. The data clearly indicate, at least according to three observations made in two different laboratories, that the gas phase of tobacco smoke, containing both combustion products and volatile agents extracted from the tobacco, produces lung tumors in mice (Leuchtenberger and Leuchtenberger 1971; Witschi et al. 1997a; Witschi 2005c). It is notable that, when cigarettes equipped with filters designed to eliminate most of the tar from inhaled cigarette smoke inhaled by humans, the expected reduction in lung cancer rates was not nearly as dramatic as had originally been hoped for. This inconsistency may have resulted from use of cigarette smoke condensate in the evaluation of cytotoxic, mutagenic, and carcinogenic properties of cigarette mainstream and sidestream smoke or of potentially less active cigarettes (IARC 2004; Wagner et al. 2000; Stratton et al. 2001), suggesting that in evaluations of modified tobacco products attention also should be directed towards the gas phase.

In addition to these practical considerations such as reproducibility, small group size and short assay duration, there is another advantage conducive to potentially fruitful research. Much
basic information on mouse lung tumors is already available. The cells of origin of the tumors are mostly type II alveolar cells and possibly Clara cells. Some cell lines have already been established in vitro and allow detailed study of pathways of signal transduction and other molecular mechanisms. The molecular biology of murine lung tumors bears many similarities to human lung cancers (Malkinson 1998; Kurie et al. 2004) and the role of inflammation, a critical event in tobacco smoke induced damage to the lung, has been thoroughly studied in the pathogenesis of these tumors (Malkinson 2005).

Because tobacco smoke is unequivocally a pulmonary carcinogen, although a comparatively weak one, it would be helpful to know to what extent strain A mice respond to other inhaled carcinogens. Unfortunately, only a few inhalation studies are available and they give conflicting results. Carcinogens such as bis(chloromethyl) ether, urethane, 1, 2-dibromoethane, ethylene oxide, and vinyl chloride were clearly tumorigenic, whereas chloromethyl methyl ether, carbon disulfide, or naphthalene, an agent positive in a 2-year bioassay, gave negative results (Adkins et al. 1986; Leong, Macfarland, and Reese 1971). Inconclusive results were obtained with diesel exhaust (Pepelko 1984). Ozone was even more ambiguous: in two early studies, there was suggestive evidence that ozone would be a lung carcinogen in strain A mice (Hassan et al. 1985; Last et al. 1987). No positive response was later observed in a study adopting the tobacco smoke protocol, i.e., exposure for 5 months, followed by a 4-month recovery period in air (Witschi et al. 1999). In a life-time bioassay, ozone was found to be a pulmonary carcinogen in female, but not in male B6C3F1 mice (Herbert et al. 1996).

There are several other disadvantages to the model, some real, some perceived. The most important shortcomings is that in general the number of lung tumors induced by tobacco smoke is comparatively small, even if the animals are exposed to high concentrations (up to 200 mg/m³ or more particulate matter). Because the metric response is lung tumor multiplicity—the average number of tumors per lung, including non-tumor bearing animals ± SD or SEM—assays with weak carcinogens have inherently limited statistical power, unless a very large number of experimental animals per group are used. When the assay was used to study the effects of several chemopreventive agents, it was found that such agents as phenethyl isothiocyanate, benzyl isothiocyanate, Bowman-Birk protease inhibitor, green tea, N-acetylcysteine, d-limonene, dexamethasone, and myo-inositol generally reduced lung tumor multiplicities by 10% to 20% (Witschi 2005c). In human clinical trials, a reduction of lung tumor rates by 20% by any chemopreventive agent could be considered a success. In the A/J mouse studies, the small differential in tumor multiplicity, after treatment with chemopreventive agents, typically a reduction from 2.2 to 2.4 tumors to 1.7 to 1.9 tumors per lung with a standard deviation of around 1.0 to 1.5, t test statistics failed to indicate significance. To obtain statistical significance in the lung tumor assays as conducted, it might take well over 100 animals per group in order to detect a significant difference at a level of p < .05 with a power of 0.7 to 0.9, considered to be a reasonable value of power for these kinds of studies (Mann et al. 1991). This limited statistical power of the assay, due to the very weak carcinogenic potency of tobacco smoke, will make it difficult to correctly interpret test results. On the other hand, if strain A mice are injected intraperitoneally with a tobacco smoke carcinogen, such as NNK or benzo(a)pyrene, 4 to 6 months later multiple tumors (20 to 40) develop. Under these circumstances, the efficiency of the same chemopreventive agents has often been quite convincingly demonstrated (Hecht et al. 2001).

Another critique relates to the observation that practically all strain A mouse lung tumors are located in the lung periphery. Most of them are benign adenomas, although some of them will progress to adenocarcinomas. Mouse lung tumors only rarely invade the adjacent tissue. As summarized by Shimkin and Stoner (1975), previous researchers found that distant metastases had developed in only 3.6% of more than 5000 mice with lung tumors. In mice exposed for lifetime to high concentrations of tobacco smoke, whereas 45% of all animals carried a benign or malignant neoplasm, nevertheless one third of the animals carried only a benign neoplasm (Hutt et al. 2005). In a similar rat study the rate of malignant lung neoplasms in females was 4.9% and of benign ones 8.6% (Mauderly et al. 2004). The histological features of murine lung tumors contrast strongly with the histological picture displayed by most human lung cancers. They most often originate from the epithelial lining of the airways; peripherally developing tumors, i.e., bronchioloalveolar carcinomas are much less frequent. Furthermore, human lung tumors metastasize aggressively to distant organs, frequently brain and liver. Lung tumors in 1-year-old mice, the time when most strain A assays are terminated, represent an early stage of progression from hyperplasia to adenoma to adenocarcinoma. It appears that a progression from benign to malignant neoplasm in both rats and mice never reaches 100% of all tumors, even at the end of the life span.

Strain A mice have a much reduced weight gain while being exposed to tobacco smoke. If killed immediately after smoke exposure, they do not carry more tumors than do control animals kept in air. However, if given a recovery period in air, tumors develop rapidly and, 4 months after cessation of smoke exposure, are present in higher numbers than in controls (Witschi et al. 1997b). It has been occasionally maintained that it is actually the postexposure weight gain that might be a determinant factor in tumor development. In a study that included mice put on dietary restriction in order to mimic the reduced weight gain during tobacco smoke exposure, it was concluded that the body weight depression during tobacco smoke exposure or, for that matter, caloric restriction with subsequent refeeding, were unrelated to the following tumor development (Stinn et al. 2005). Swiss-Webster mice, exposed to tobacco smoke, gain the same weight as do their air controls, and still develop more lung tumors (Witschi et al. 2002).

Some additional considerations apply to the strain A mouse lung tumor assay. The strain A mouse lung is exquisitely
sensitive to a wide variety of carcinogens and any genotoxic carcinogen is prone to produce lung tumors, regardless of route of administration (inhalation, oral, intraperitoneal). Although polycyclic aromatic hydrocarbons, nitrosamines, and carbamates reliably produce a strictly dose-dependent increase in lung tumors in strain A mice, the animals do not, if at all, respond to certain other classes of carcinogens such as aromatic amines or metals. Originally, it was hoped that the strain A mouse lung tumor assay could serve as an efficient short-term carcinogenesis bioassay (Shimkin and Stoner 1975), but a later validation assay gave less encouraging results (Maronpot et al. 1986).

THE CHALLENGE TO TOXICOLOGY

In 1973, one of the keynote speakers at the First International Congress of Toxicology and later recipient of the Society of Toxicology’s Merit Award, W. N. Aldridge (for biography see Johnson 2001) challenged the science and practitioners of toxicology by saying: “To rely on larger and larger experiments involving more and more animals is a gesture of defeat.” Has this challenge been met in the attempts to develop an animal model of tobacco smoke carcinogenesis? Or has defeat already tacitly been accepted?

Tobacco smoke-induced tumors in the larynx of hamsters—although not in the deep respiratory tract—had been observed as early as 1973 (Dontenwill et al. 1973). These experiments involved 4440 hamsters. What is now considered to be conclusive evidence for tobacco smoke–induced lung carcinogenesis in rats and mice was only obtained recently. A rat study involved a total of 753 male and female F344 rats and two exposure doses (low and high), applied over 30 months. Life-time exposure to tobacco smoke produced a significant increase in lung tumors in females in the high-dose group, where tumor rates were 14% versus 0% in controls. In male rats, only a statistically nonsignificant increase (9% versus 2%) was recorded (Mauderly et al. 2004). In a study with female B6C3F1 mice, where animals were exposed up to 930 days, a statistically much more robust increase in lung tumor rates (malignant and benign combined) was found: a rate of 45% in 330 smoke exposed animals as opposed to 10% only in 326 controls (Hutt et al. 2005). The study was called the first example of a strong carcinogenic response in the lungs of animals exposed to cigarette smoke. Moreover, the model was recommended for future testing and evaluation of modified tobacco products for chemoprevention studies (Hecht 2005), although this success has come at the expense of considerable resources.

During the past several decades, great advances in mechanistic toxicology have been made. There is certainly no lack of such data on tobacco smoke and lung cancer (Hecht 1999, 2002; De Flora et al. 2003a). In addition, several short-term animal bioassays were also developed in the hope to replace 2-year bioassays. They included not only the strain A mouse, but breast tumors in female Sprague-Dawley rats, hepatocyte foci in rat liver, skin painting studies in SENCAR mice, and more lately transgenic mouse models. A plethora of in vitro assays is also available. At present, it would not seem that any of these approaches has been deemed to be useful for evaluation of tobacco smoke carcinogenesis, with the possible exception of skin painting studies (Curtin et al. 2004a). If future development of modified tobacco products and identification of chemopreventive agents will depend mostly on the use of experiments approximating the “megamouse study” and tumors will be the only acceptable biomarker of effect, then one has to wonder about the impact of mechanistic toxicology. Although we have multiple and quite refined tools, we often do not trust them to be predictive. Positive mechanistic data, including results from in vitro system, often help to decide whether a given product shall or shall not be developed further. Negative mechanistic data are at best used only to substantiate claims for “safety” obtained in whole animal bioassays.

It might be worthwhile to remember how pathology, often described as “descriptive,” actually became not only a diagnostic, but also a predictive tool. During the golden age of pathology, about 1850 to 1950, pathologists routinely looked at disease in all its stages, be it because people died from the disease or with the disease. The accumulated body of information eventually allowed one to look at a lesion at one time point while knowing, based on experience, how it had looked in the past and how it would look in the future. Old pathology texts describe disease processes in great detail and with graphic examples. More recently, tools of modern molecular biology have been used to follow over time—and thus eventually predict—sequential genetic alterations during the development of colon polyps or of neoplastic changes in the respiratory tract (Wistuba and Gazdar 2003). But there are very few studies in toxicology that would have followed this approach.

Toxicology, steeped in and committed to the traditions of pharmacology (dose response) and biochemistry (the biochemical lesion), has paid little attention to the evolution of lesions with time. An excellent case that this should be done has recently been made by Rozman et al. (2006). They emphasize toxicokinetics and toxicodynamics. Their considerations and insights need a broader analysis of the biological response and how it may me defined over time. The challenge to toxicology will be to develop confidence into the predictive power of early events by following their development over time, as was done in pathology. Eventually, we then will no longer have to rely on the terminal phenotype that, so far, can only be obtained in expensive and intellectually little challenging long-term studies. It will be even more important to make a convincing case about the validity and acceptability of predictive studies to the stakeholders at large. The old Paracelsian paradigm “the dose makes the poison” should be amended to “The dose makes the poison, but time will determine the final result.”

REFERENCES


