1. ABSTRACT

Primary pulmonary malignancies remain the major source of cancer-related deaths in the Western World. While surgical resection is an efficacious therapy for those with early stage disease, the majority of patients present with advanced malignancies and systemic treatments, such as cytotoxic chemotherapy, have only limited efficacy in lung cancer. Furthermore, chemoprevention for current or former smokers has demonstrated only limited success using available agents. The mouse model of primary lung carcinogenesis represents a very valuable tool for the study of tumor initiation, promotion, and therapy. Here we discuss several models of chemically-induced murine lung cancer with a specific emphasis on translational and clinically-relevant lines of investigation. We emphasize the pros and cons of currently available models in order to facilitate further investigations into the development and treatment of primary pulmonary malignancies.

2. INTRODUCTION

Lung cancer is the leading cause of cancer death in the United States killing an estimated 160,000 people annually with approximately 200,000 newly diagnosed in 2010 alone (1). The number of deaths caused by lung cancer exceeds that of colon, breast and prostate cancer combined. Lung cancer is associated with a dismal 5-year survival rate of 15% due to the fact that the majority of patients are diagnosed in the late stages of disease after metastasis has occurred. Human lung cancer is comprised of two main histopathologic groups, non-small cell (NSCLC) and small cell lung cancer (SCLC). Approximately 80% of lung cancers are NSCLC, originating from lung epithelial cells. NSCLC is further subdivided into adenoc, squamous, and large cell subtypes. Adenocarcinomas arise in the periphery and comprise ~40% of all NSCLC. In recent decades the prevalence of lung adenocarcinoma has been on the rise and is now the most common cancer type among women and non-
Mouse models of chemically-induced lung carcinogenesis

Smokers. Squamous cell lung cancer (SCC) accounts for ~25% of NSCLC, arises in the central airways and is most strongly associated with male smokers. The majority of remaining NSCLCs (~15%) are large cell carcinomas with a smaller percentages of mixed (e.g. adenosquamous) and undifferentiated tumors. SCLC is histologically distinct from NSCLC and originates from the neuroendocrine cells of the bronchus. SCLC makes up ~15% of lung cancers, and is associated with extremely poor prognosis because of high metastatic potential early during tumor progression. In developed nations smoking rates have dropped considerably since the 1960s. Nevertheless, cigarette smoking causes approximately 85% of lung cancer and there remain millions of individuals who currently smoke or are former smokers. These individuals have an elevated risk for developing lung cancer and are targets for chemoprevention. Additionally, smoking rates in many developing countries continue to rise and as a result lung cancer will continue to be a major global health problem for the foreseeable future.

3. MOUSE MODELS OF LUNG CANCER

The mouse is the principal animal model for the study of lung cancer. Widespread adoption of mice as models for human lung cancer is a consequence of a long history of use focused around the breadth of genetic variation, ease of genetic manipulation, and the ability to induce lung cancer with molecular and histological similarities to human disease. Mouse lung cancer models are now frequently used in pre-clinical tests of therapy and prevention. Engineered models that replicate specific genetic lesions found in human tumors, such as expression of activated oncogenes (e.g. Kras) or inactivated tumor suppressor genes (e.g. p53) are often used to investigate the genesis of lung cancer. In addition, chemical carcinogen-induced models utilizing mouse strains with a predisposition to cancer have been used to successfully address the genetic complexity of human lung tumors. Our goal in this review is to provide a summary of the current state of these carcinogen-induced models. The reader is referred to several recent reviews addressing the use of engineered mouse models for further information on genetic models of lung cancer (2-4).

In humans, complex chemical mixtures, in particular cigarette smoke, are the predominant initiator of lung cancer. Because cigarette smoking is the primary cause of human lung cancer, individual cigarette smoke carcinogens are frequently used to induce lung tumors in mice. This is commonly performed by intraperitoneal or dietary administration of carcinogens of the polycyclic aromatic hydrocarbon (PAH) and nitrosamine class (5-8). PAHs are largely produced during the combustion of tobacco, while nitrosamines are already present in unburned tobacco and are formed as a consequence of the tobacco curing process. Benzo(a)pyrene (B(a)P), a PAH, and the nitrosamines, 4- (methylnitrosamo)-1- (3-pyridyl)-1-butane (NNK) and N'-nitrosonornicotine (NNN), are strong inducers of lung adenomas and adenocarcinomas in mice. These chemicals are pro-
carcinogens that require metabolic activation to electrophilic compounds that react with DNA and form adducts. Subsequent failure of repair or misrepair results in genetic mutations. Cytochrome P450 enzymes are central to bioactivation of B(a)P and NNK, and are encoded by a variety of Cyp genes that exhibit differences in tissue expression, and chemical specificity (9). P450s are responsible for activation of B(a)P to a diol-epoxide, that reacts with deoxyguanine to form a bulky adduct, C8-B(a)P-diolepoxide-guanine, which most often results in G- to T nucleotide transversions. P450s also catalyze N-hydroxylation of NNK, which spontaneously decomposes to aldehydes and diazonium ions, subsequently forming O6- methyl-guanine adducts and ultimately G-to-T transversions (10). P450 enzymes are expressed most abundantly in the liver, but are also present in the peripheral and bronchial epithelia of the lung. Conditional, lung-specific deletion of NADPH-P450 reductase (the only mammalian P450 reductase gene and electron donor for many P450 reactions), demonstrated reduced NNK-induced tumor load concurrent with lower O6-methylguanine levels (11). In contrast, the authors demonstrated that conditional liver-specific deletion of NADPH-P450 reductase increased lung tumor burden after i.p injection of NNK. This result suggests that the overall function of liver P450 enzymes in response to NNK is detoxification and metabolism, while P450 enzymes in the lung bioactivate the NNK pro-carcinogen.

Inbred mouse strains, such as A/J and SWR, have a high incidence of spontaneous lung tumor development. A/J strain mice have roughly 80-100% incidence of spontaneous lung tumors after 24 months, and tumors are often detected within the first 6 months of age (5, 12). These strains are also very susceptible to carcinogen-induced lung tumors. Other strains such as C57BL/6/J, C3H/J and DBA are very resistant to carcinogen-induced lung tumors, while strains such as O20 and BALB/c have intermediate susceptibility. A/J strain mice develop approximately 25 tumors per lung 14-16 weeks post-treatment with the carcinogen ethyl carbamate (urethane), while the C57BL/6J strain develops less than 1 tumor per lung on average (5, 7, 13-15) (Table 1). These strain-dependent differences have permitted several research groups to map genetic susceptibility loci that associate with both carcinogen-induced and spontaneous lung tumor development (16, 17).

Muirne lung tumors bear similar morphology, histopathology, and molecular anomalies as those observed in human tumors. The majority of tumors observed in murine models are benign pulmonary adenomas that have clear borders and are comprised of well-differentiated cells. Although adenomas are rarely observed in humans, likely because they are asymptomatic and not frequently diagnosed, murine adenomas do exhibit histological similarity to non-small cell lung adenocarcinoma derived from airway type II cells. Murine adenomas are considered precursors to murine lung adenocarcinomas as they do progress to malignant adenocarcinomas of various subtypes (solid, papillary, bronchiolo-alveolar) which show signs of nuclear atypia and invasiveness (18).
Table 1. Selected rodent models of chemically-induced lung carcinogenesis

<table>
<thead>
<tr>
<th>Model</th>
<th>Strain</th>
<th>Carcinogen</th>
<th>Tumor</th>
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<tbody>
<tr>
<td>Mouse AD/ADC</td>
<td>A/J</td>
<td>B(a)P, i.p. 100 mg/kg</td>
<td>20 w: 8-10 tumors (AD), 100% incidence (20, 21)</td>
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<td>B(a)P, i.g. 100 mg/kg (3X)</td>
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<td></td>
<td>A/J</td>
<td>NNK, i.p. 100 mg/kg</td>
<td>20 w: 6-8 tumors (AD), 100% incidence (20, 22, 23)</td>
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<td>52 w: 15 tumors (95% AD, 5% ADC), 70-80% incidence (ADC)</td>
</tr>
<tr>
<td></td>
<td>A/J</td>
<td>Urethane, i.p. 1 g/kg</td>
<td>16 w: 20-25 tumors (AD) (21, 24-26)</td>
</tr>
<tr>
<td></td>
<td>A/J</td>
<td>Vinyl carbamate, i.p. 60 mg/kg</td>
<td>24w: 25 tumors (AD), 12% incidence (ADC)</td>
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<td>52 w: 30% incidence ADC (27, 28)</td>
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<tr>
<td>Swiss albino</td>
<td></td>
<td>Main-stream cigarette smoke, 120 days</td>
<td>26-33w: 6-14 tumors (AD), 80% incidence (AD), 5-20% incidence (ADC) (29)</td>
</tr>
<tr>
<td></td>
<td>A/J</td>
<td>Main- and side-stream cigarette smoke, 5 mos smoke + 4 mos air</td>
<td>3 tumors (AD) vs 1 spontaneous tumor (AD) (30)</td>
</tr>
<tr>
<td></td>
<td>B6C3F1</td>
<td>Mainstream cigarette smoke, lifetime</td>
<td>10X increase in hyperplasia, 4.6X AD and papilloma, 7.3X ADC, 5X metastatic pulmonary ADC (31)</td>
</tr>
<tr>
<td>Rat AD/ADC</td>
<td>F344</td>
<td>NNK, s.c. 1.5 mg/kg (3X, 20 w)</td>
<td>98w: 67% incidence (AD), (33% ADC) (32)</td>
</tr>
<tr>
<td></td>
<td>F344</td>
<td>Mainstream cigarette smoke, up to 30 months</td>
<td>Incidence increased from 0% in control to 6% (light smoke) to 14% (heavy smoke) (33)</td>
</tr>
<tr>
<td>Mouse squamous</td>
<td>Swiss ~ 8 w</td>
<td>NTCU, 3 µmol, 2x week (22 w)</td>
<td>24w: 50% hyper/metaplasia, 10% CIS/SCC (34)</td>
</tr>
</tbody>
</table>

i.p. = intraperitoneal, i.g. = intragastric, i.t. = intratracheal, AD = adenoma, ADC = adenocarcinoma

Murine models of lung carcinogenesis exist for adenocarcinoma and squamous cell subtypes (Table 1), while currently those for small cell carcinoma rely solely on genetic ablation of Rb and p53 genes, and large cell models have yet to be described. The majority of mouse lung adenoma/adenocarcinoma studies employ single intraperitoneal injection of the carcinogens B(a)P, NNK, urethane or vinyl carbamate in a susceptible strain such as A/J at 5-6 weeks of age. At 20 weeks post injection these carcinogens cause anywhere from 8-25 tumors of which almost 100% are histologically adenomas. At 52 weeks post-initiation, 1-2 adenocarcinomas are typically observed.

Most lung carcinogens are ‘complete’ and thus both initiate tumorigenesis and promote tumor progression through dysregulated proliferation of Clara cells and Type II pneumocytes. Soon after initiation, hyperplastic foci in the bronchioles and alveoli are observed. Which of these foci progress to adenomas, and which of these spontaneously regress is currently unknown. However, progression of adenoma to invasive adenocarcinoma may be infrequent (< 10%, 1 year post carcinogen) and metastasis to other organs is extremely rare in carcinogen-based models (19). Thus, the majority of tumors remain as benign adenomas even 1 year post initiation of carcinogen. Immunohistochemical staining of tumors is typically positive for surfactant protein C (SPC, an immunohistochemical marker for type II cells), but not secretoglobin 1a1 (also known as Clara cell secretory protein (CCSP) or CC10, a marker for Clara cells), suggesting that most mouse lung adenoma/adenocarcinomas originate from Type II cells, or potentially through loss of expression of CC10 in a Clara cell transdifferentiation process.

Activating mutations in Kras are a prominent early events observed in both human and mouse lung tumors induced by carcinogen (35, 36). The human precursor lesion to adenocarcinoma, atypical adenomatous hyperplasia (AAH), possesses Kras mutations at similar frequency to adenocarcinomas, suggesting that the Kras mutation is an important early event in tumorigenesis (37). Estimates are that 15-50% of all human lung adenocarcinomas possess mutations in Kras, most commonly in codon 12, and less often in codons 13 and 61 (38-40). The tumor suppressor genes Trp53, p16, Rb, Apc, and p16\(^{\text{INK4a}}\) (Cdkn2a) are also suppressed and/or inactivated in both human and mouse tumors, either through methylation or less frequently by mutation, suggesting that the molecular mechanisms between species are relatively well conserved (18, 41).

In mouse, factors influencing Kras mutational frequency and spectrum include the type of carcinogen, the type of adduct formed, age of the mouse (fetal or adult), dose of carcinogen and strain susceptibility. In the A/J mouse, activating mutations in Kras are observed in 100% of chemically-induced lung tumors and in greater than 80% of spontaneous tumors (21, 42). Kras mutations in humans are typically G-T transversions and correlate with smoking. Similarly, Kras mutations in mouse lung tumors are G-T transversions in codon 12 when initiated by the cigarette smoke carcinogens B(a)P or NNK. Alternatively, urethane and vinyl carbamate most often cause codon 61 A-T transversions in A/J mice.

Approximately 60 known carcinogens are present in both mainstream and sidestream cigarette smoke, in addition to several co-carcinogens and tumor promoting compounds. Initial attempts using cigarette smoke to produce pulmonary malignancies with high incidence in mice indicated cigarette smoke was weakly tumorigenic in mouse models (43). Subsequently, a protocol was developed by which tumors could be induced in A/J strain mice through a 5 month exposure of a combined mainstream and sidestream smoke followed by a 4 month exposure to normal air (30). This exposure to air was essential to the development of tumors. Multiple groups have used this protocol (generally in A/J and SWR strains) and have observed an increase in tumor multiplicity from
progression of tumorigenesis can be accelerated by chronic activation of proto-oncogene K-Ras. Subsequent promotion and progression of tumorigenesis can be accelerated by chronic administration of non-carcinogenic lung inflammatory agents, such as the chemical butylated hydroxytoluene (BHT) (50-52). BHT undergoes metabolism by lung specific P450s to a very reactive BHT-quinone methide, which subsequently forms adducts with cellular proteins and creates an environment of chronic tissue damage and compensatory epithelial cell proliferation. This includes type II cell necrosis followed by type II cell hyperplasia and differentiation to replace lost type I cells (53-55). Repeated delivery of BHT causes massive inflammatory cell infiltration in the alveolar spaces of inflammation susceptible BALB/cByJ (BALB) strain mice. Importantly, if BHT is administered weekly for 6 weeks after an initial single dose of MCA, the result is a 10-fold enhancement in observed lung tumors (54, 55). It is important to note that there is strong genetic control of these inflammatory and tumor responses, as different inbred mouse strains differ in the degree of inflammation and degree of tumor promotion caused by BHT. C57BL/6J (B6) strain mice exhibit low levels of BHT-induced inflammation and are also resistant to tumor promotion by BHT, while strains such as A/J and BALB are considered susceptible. BHT elicits similar injury as other lung irritants, such as ozone, crystalline silica, hyperoxia, and vanadium pentoxide (50). Several genetic susceptibility mapping studies have identified genetic loci for susceptibility to inflammation that are common to different lung inflammatory agents, suggesting they operate by similar mechanisms. Interestingly, many of these loci overlap known lung cancer susceptibility loci, suggesting common mechanisms of action between lung injury/inflammation and carcinogenesis (56).

Recent studies have demonstrated that neutrophils play a seminal pro-tumorigenic role in mediating tumor promotion by BHT (57). When compared to tumor promotion in control IgG-treated BALB mice, antibody-mediated depletion of neutrophils in BALB/cByJ mice, reduced tumor multiplicity by 71%. BHT induces both neutrophil numbers and levels of the neutrophil chemokine KC in the airways of susceptible BALB/cByJ mice. Furthermore, data from this study suggests that KC expression by lung tissue-resident CD11c+ cells may play an important role in susceptibility to pulmonary carcinogenesis by maintaining high levels of neutrophil trafficking into the lung. This is consistent with other studies showing similar kinetics of KC and neutrophil levels, caused by V_{2}O_{5}, another tumor promoter of MCA tumorigenesis (58). The requirement for neutrophils in this model is consistent with enhanced neutrophil and KC levels observed in bronchoalveolar carcinoma patients with poor outcome (59). These data indicate that neutrophils and their effector functions are potential targets for prevention and therapy.

5. LUNG CANCER CHEMOPREVENTION IN RODENT MODELS OF CHEMICAL CARCINOGENESIS

Chemically-induced lung tumors have been widely used to identify drugs and botanically-derived agents that may be effective for chemoprevention. Chemoprevention can be defined as the use of chemo- or...
Mouse models of chemically-induced lung carcinogenesis

dietary agents to prevent tumor formation or progression. As mentioned above, chemically-induced lung tumors in the mouse share many characteristics with human lung cancer, both genetic and histological. These properties also make them a suitable model to use for chemoprevention. Administration of a test compound can begin anywhere from pre-initiation to late in the tumorigenesis process. Because the stages of lung tumor progression are well characterized in chemically induced mouse lung tumors, the efficacy of chemo- or dietary agents can be determined at each step in the tumorigenic process, an important consideration for translating findings into humans.

Because of its susceptible nature, the most common pre-clinical chemoprevention model for the lung is the A/J strain mouse. The cigarette smoke carcinogens B(a)P and NNK have been the most widely used and are typically administered by i.p. injection, although other carcinogens such as urethane and vinyl carbamate and other routes of administration such as oral gavage have been used. The types of chemoprevention schedules used can be divided into three general categories: 1) complete, where the agent is administered beginning before carcinogen administration and continues throughout the experiment; 2) initiation, where the agent is administered from just before carcinogen administration and is terminated within a few weeks of initiation, and 3) progression, where treatment is begun after carcinogen administration and continued until experiment termination. Obviously, there are many variations of experimental design that will depend on the goals of the investigators.

To date, over 200 pre-clinical studies of lung cancer chemoprevention have been published and it is beyond the scope of this brief review to begin to discuss them all. An ideal chemoprevention agent will combine efficacy with a very favorable safety profile. Because chemoprevention agents would be administered to patients essentially free of overt disease, the presence of even relatively mild effects could limit the usefulness of a compound due to low levels of patient compliance as well as concerns over patient welfare. This is a major reason why many chemoprevention studies have focused on using botanically derived agents, sometimes referred to as nutraceuticals. These include both complex mixtures isolated from a given plant type (e.g. tea polyphenol fractions or freeze dried berries) and purified compounds that are thought to be the main active ingredients present in these preparations. The compound (-)-epigallocatechin gallate (EGCG) is generally considered to be the most potent polyphenolic compound in green tea extracts. However, chemoprevention with purified EGCG has not yielded as large of an effect as the complex mixture (60). The use of complex mixtures may provide higher degrees of efficacy or alternatively improve stability and bioavailability.

The timing of administration of a chemopreventive agent is of great importance when testing a new compound. To be useful in humans, a chemoprevention agent must effectively block or slow the progression of pre-cancerous lesions to cancerous ones. In many early studies, the administration of a chemopreventive agent began prior to initiation with the carcinogen. This raises the possibility that the effect of the agent is on expression of metabolic enzymes, agent uptake or agent excretion rather then effects on tumor progression per se. Endpoints such as incidence, multiplicity and tumor size are frequently measured. In addition, the pathology and histopathology of lesions is frequently investigated. The detection and localization of cells expressing a particular protein in lung tumors can provide clues as to molecular mechanisms of the agent and has the potential for identifying biomarkers.

Several notable limitations for testing chemoprevention agents in mouse models exist. As mentioned above, beginning treatment prior to initiation of carcinogenesis has the potential to cause changes in tumor formation that are based on alterations in carcinogen metabolism. Notable differences in key metabolic enzymes such as cytochrome p450s exist between mice and humans and could ultimately affect the disposition of the carcinogen. This is another reason to avoid starting chemoprevention prior to initiation of carcinogenesis. The metabolic differences between rodents and humans can of course also have effects on the metabolism and ultimate excretion of preventive agents. The route of administration of a chemoprevention agent can also have a major effect on the bioavailability of that agent. Ideally, pharmacokinetic/phamacodynamic studies should be performed in conjunction with initial characterization of a chemopreventive agent.

6. CONCLUSIONS

Mice have been, and will continue to be, a valuable resource in the modeling of human lung neoplasms. Critiques of the models discussed is based mainly on the predominant use of large doses of single carcinogens that far exceed the human situation in which the carcinogen dose is accumulated over time, and via a mixture of carcinogens through cigarette smoke. Particularly promising in addressing this issue is the development of cigarette smoke-induced models that has hastened over the last decade. Nevertheless, mouse models of lung carcinogenesis as a whole have been seminal to the discovery of etiological factors of disease, and are now frequently used to assess efficacy of various treatments and preventive agents that may benefit the human condition.

7. REFERENCES

Mouse models of chemically-induced lung carcinogenesis


944
Mouse models of chemically-induced lung carcinogenesis


Mouse models of chemically-induced lung carcinogenesis


Key Words: Carcinogen, Adenoma, A/J strain, Chemoprevention, Review

Send correspondence to: Haris G. Vikis, Department of Pharmacology and Toxicology, Medical College of Wisconsin and MCW Cancer Center, Milwaukee, WI, 53202, Tel: 414-955-7588, Fax: 414-955-6059 E-mail: Haris Vikis, hvikis@mcw.edu