Alterations in the K-ras and p53 Genes in Rat Lung Tumors

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Activation of the K-ras protooncogene and inactivation of the p53 tumor suppressor gene are events common to many types of human cancers. Molecular epidemiology studies have associated mutational profiles in these genes with specific exposures. The purpose of this paper is to review investigations that have examined the role of the K-ras and p53 genes in lung tumors induced by the F344 rat by mutagenic and nonmutagenic exposures. Mutation profiles within the K-ras and p53 genes, if present in rat lung tumors, would help define some of the molecular mechanisms underlying cancer induction by various environmental agents. Pulmonary adenocarcinomas or squamous cell carcinomas were induced by tetranitromethane (TNM), 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK), beryllium metal, plutonium-239, X-ray, diesel exhaust, or carbon black. These agents were chosen because the tumors they produced could arise via different types of DNA damage. Mutation of the K-ras gene was determined by approaches that included DNA transfection, direct sequencing, mismatch hybridization, and restriction fragment length polymorphism analysis. The frequency for mutation of the K-ras gene was exposure dependent. Only two agents, TNM and plutonium, led to mutation frequencies of > 10%. In both cases, the transition mutations formed could have been derived from deamination of cytosine. The identification of non-ras transforming genes in rat lung tumors induced by mutagenic and nonmutagenic exposures such as NNK and beryllium would help define some of the mechanisms underlying cancer induction by different types of DNA damage. Altaration in the p53 gene was assessed by immunohistochemical analysis for p53 protein and single-strand conformation polymorphism (SSCP) analysis of exons 4 to 9. None of the 93 adenocarcinomas examined was immunoreactive toward the anti-p53 antibody CM1. In contrast, 4 of 71 squamous cell carcinomas exhibited p53 immunoreactivity with no correlation to type of exposure. However, SSCP analysis only detected mutations in 2 of 14 squamous cell tumors that were immunoreactive, suggesting that protein stabilization did not stem from mutations within the p53 gene. Thus, the p53 gene does not appear to be involved in the genesis of most rat lung tumors — Environ Health Perspect 105(Suppl 4):901–906 (1997)

Key words: K-ras, p53, rat lung tumors, NNK, beryllium, plutonium, X-ray, diesel exhaust, carbon black

Introduction

Activation of the K-ras protooncogene and inactivation of the p53 tumor suppressor gene are events common to many types of human cancers (1,2). Molecular epidemiology studies have associated mutational profiles in these genes with specific exposures. For example, lung tumors from cigarette smokers (3) or induced in mice by exposure to polycyclic aromatic hydrocarbons (4) contain a GGT → TGT transversion mutation in codon 12 of the K-ras gene. Further evidence that some carcinogens may produce a specific and recognizable pattern of gene mutations has been demonstrated by mutational hot spots in the p53 gene in liver and skin tumors associated with exposure to aflatoxin (5) and ultraviolet radiation (6), respectively. In all these cases, the specific mutation could be ascribed to the type of DNA damage produced by the exposure.

Lung cancer attributable to tobacco use is the leading cause of cancer-related death in the United States (7). However, only 16% of habitual smokers develop lung cancer, suggesting that host susceptibility and other environmental factors (e.g., air pollution) contribute to the risk of developing this disease. The best example of a combined exposure increasing lung cancer risk can be seen in underground uranium miners who were highly exposed to radon progeny in conjunction with tobacco use. Their lifetime risk for developing lung cancer is > 50% (8). Chronic low-level inhalation of radon in the home has also been estimated to contribute to 10% of all lung cancer deaths in the United States (8). The effect of exposure to other types of environmental pollutants (e.g., diesel exhaust, ionizing radiation) may also be an added risk factor for lung cancer (9).

The F344 rat has been used as a model to evaluate the carcinogenic potential of chronic exposure to environmental chemicals or mixtures. Several exposures have resulted in a significant increase in primary lung tumors. Mutation profiles within the K-ras and p53 genes, if present in rat lung tumors, could help define some of the molecular mechanisms underlying cancer induction by these environmental agents. The establishment of distinct mutational profiles within these genes for a specific environmental agent would also improve risk estimates from exposures in the home and workplace. The purpose of this paper is to review investigations that have examined the role of the K-ras and p53 genes in lung tumors induced in the rat by mutagenic (genotoxic) and nonmutagenic (nongenotoxic) exposures. Lung tumors were induced by tetranitromethane (TNM) (10), 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK)(11), beryllium metal (12), plutonium-239 (13), X-ray (14), diesel exhaust, or carbon black (15). These exposures were chosen because the tumors produced could arise via different types of DNA damage. TNM can stimulate cell proliferation by its irritant
properties and cause deamination of DNA (10). NNK is a tobacco-specific nitrosoamine that is genotoxic to DNA through the generation of alkylating (11) and pyridylloxobutylating agents (16). Beryllium metal is a nongenotoxic carcinogen that elicits a chronic inflammatory response involving the recruitment of macrophages and neutrophils that can release cytokines and oxygen radicals (12). Plutonium-239 and X-ray exposures damage DNA directly and through the production of free radicals (17). Diesel exhaust contains 8% particle-associated, extractable, organic compounds by weight in conjunction with the insoluble particles within the carbon matrix, thereby not only producing genotoxic effects on DNA, but also causing chronic inflammation and epithelial proliferation (15). Carbon black contains only 0.12% extractable organics and serves as a surrogate for the elemental matrix of diesel exhaust soot (15).

Materials and Methods

Exposure and Sampling

The exposures have previously been described in detail (10–15). All exposures except NNK were by the inhalation route using respirable aerosols. NNK was administered by sc injection. Lung neoplasms were fixed in 4% buffered paraformaldehyde or in 10% neutral buffered formalin. These samples were embedded in paraffin, cut at 5 μm, stained with hematoxylin and eosin, and examined by light microscopy for histologic diagnosis. Serial sections were also cut for immunohistochemical assays and DNA analysis. If lung tumor specimens were >0.15 mg, the additional tissue was frozen in liquid nitrogen and stored at −80°C. DNA was isolated from fixed and frozen tissue for the analyses described below using standardized techniques (12,18).

Analysis of Mutations in the K-ras Gene

The first and second exons of the K-ras gene, specifically codons 12, 13, and 61, contain the majority of activating mutations identified in human and murine tumors (1). Several methods for detecting mutations within this gene have been used over the past 10 years and have been described previously (10,12,14,18–22). These methods include DNA transfection, oligonucleotide mismatch hybridization, direct sequencing of exons 1 and 2 following amplification by the polymerase chain reaction, and BstN1 restriction fragment length polymorphism assay. In all the present studies described, two independent assays were used to confirm the detected mutations.

Analysis of Mutations in the p53 Gene

Two screening methods were used to detect evidence for alterations in the p53 gene. First, immunohistochemical analysis of the p53 protein, which relies on the nuclear localization and increased stability of mutant p53 proteins, was used to identify cells with altered expression of the p53 gene (12). Second, to identify the exon containing the possible mutation, exons 4 to 9 of the p53 gene were screened for mutations by single-strand conformation polymorphism (SSCP) analysis (18); >90% of the mutations in p53 are localized within these exons (2). Finally, mutations were identified by direct sequencing of exon-specific polymerase chain reaction products (12).

Results

K-ras Mutations in Rat Lung Tumors

The frequency for mutation of the K-ras gene was exposure dependent, but was not associated with histologic tumor type. Mutations detected were present at similar frequencies in adenocarcinomas and squamous cell carcinomas. Results are summarized in Table 1. All lung tumors induced by TNM contained a GGT → AGT transition mutation within codon 12 of the K-ras gene (10). In marked contrast, no activating mutations were present in lung tumors induced by NNK (19). Mutation frequencies of 6 and 10% were detected in tumors associated with carbon black and diesel exhaust exposure, respectively. Only 10% of tumors induced by beryllium metal contained a K-ras mutation; in both cases a GGT → GTT transversion was observed (12). Lung tumors induced by plutonium showed a higher frequency for mutation of the K-ras gene than tumors induced by X-rays; 46% of tumors induced by plutonium contained a mutation within this gene, predominantly a GGT → AGT transition in codon 12 (20). In contrast, only 3% of X-ray-induced tumors contained an activating mutation (14). No mutations were detected in the four sham-exposed lung tumors.

A BstN1 enrichment assay, which can detect one mutant allele in 10⁴ copies of wild-type allele, was required to detect the mutations observed in the beryllium- and X-ray-induced tumors. All other mutations were detected without enriching for the mutant allele. Thus, the activation of the K-ras gene in the beryllium- and X-ray-induced tumors is both a rare and a late event, possibly stemming from genomic instability during tumor progression.

p53 Mutations in Rat Lung Tumors

All lung tumors, except those induced by TNM (not available for analysis) were examined immunohistochemically for the p53 protein. A sarcoma induced in the nude mouse by the SV40 immortalized rat 2 cell line showed strong immunoreactivity toward the anti-p53 antibody CM1 and was used as a positive control (12). Positivity for p53 protein was associated with tumor histology. None of the 93 adenocarcinomas examined (all exposures) were immunoreactive with the p53 antibody. In contrast, 14 of 71 squamous cell carcinomas exhibited nuclear p53 immunostaining as summarized in Table 2. Immunoreactivity was detected in 57 and 50% of squamous cell carcinomas induced by diesel exhaust or carbon black (18), albeit with low sample sizes (n = 7 and 4), while only 18 and 7% of tumors induced by X-rays (14) or plutonium (21), respectively, contained aberrant p53 protein. Only 18% of squamous cell tumors

Table 1. Pattern and frequency of K-ras gene mutations in rat lung tumors.

| Exposure   | Frequency | %   | Activating mutation |
|------------|-----------|-----|---------------------|
| Sham       | 0/4       | 0   | —                   |
| TNM        | 19/19     | 100 | Codon 12: GGT → GAT |
| NNK        | 0/21      | 0   | Codon 12: GGT → GTT |
| Beryllium  | 2/24      | 10  | Codon 12: GGT → GAT |
| Diesel exhaust | 2/21 | 10  | Codon 12: GGT → GAT |
| Carbon black | 1/18 | 6   | Codon 12: GGT → GTT |
| Plutonium  | 33/71     | 46  | Codon 12: GGT → AAT |
| X-ray      | 1/35      | 3   | Codon 12: GGT → GAT |
induced by NNK exhibited nuclear p53 immunostaining. No squamous cell carcinomas were available from the beryllium carcinogenesis study.

The immunostained nuclei in the squamous cell carcinomas were generally distributed throughout the neoplasm in the basilar layers of the neoplastic cords and in the poorly differentiated portions of the neoplasms (Figure 1A, B). The more differentiated polyhedral, keratinizing cells toward the centers of the neoplastic cords showed little to no nuclear reactivity (14,18,21,22). Immunoreactivity varied among tumors; it was strong in intensity in multiple foci of some tumors while more focal, intense staining was observed in other tumors. In some tumors staining was diffuse, but weak in intensity (14,18,21,22).

Because immunohistochemical analysis of the p53 protein does not detect alterations that result in the loss of protein expression, nonsense mutations, or missense mutations outside of exons 5 to 8, SSCP analysis of exons 4 to 9 was conducted on all tumors. No mutations were detected in any of the adenocarcinomas. In spite of the immunoreactivity observed in the 13 squamous cell carcinomas available for screening, SSCP analysis (Table 2) only detected mutations in one of the NNK-induced tumors (exon 5) and both plutonium-induced tumors (exon 8). Two additional tumors contained mutations: one was a silent mutation within exon 8 from a diesel exhaust-induced tumor, and the other was found in exon 9 from an X-ray-induced tumor. Three of the mutations were G→A transitions, while the fourth mutation was a T→C transition (Table 2, Figure 2).

**Discussion**

The results from these investigations indicate that the involvement of the K-ras and p53 genes in the genesis of rat lung tumors is specific to the exposure material and the resulting histologic form of the tumor, respectively. The only two exposures that led to activation of the K-ras gene at frequencies >10% were that of TNM and plutonium. In both cases, the mutations formed could have stemmed from deamination of cytosine. Nitro-containing compounds such as TNM can cause subsequent base mispairing by deaminating a base such as cytosine (10).

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**Table 2. Frequency for alteration of the p53 gene in rat squamous cell carcinomas.**

| Exposure    | Immunostaining Frequency | SSCP Analysis Frequency | Mutation                      |
|-------------|--------------------------|-------------------------|-------------------------------|
| Sham        | 1/2 50 ND                |                         | Exon 5, codon 143: TTG→TCG   |
| NNK         | 2/11 18 1/11 9           |                         | Exon 8, codon 722: GTT→GTG   |
| Diesel exhaust | 4/7 57 0/4 0        |                         | Exon 9, codon 309: AGC→AAC   |
| Carbon black | 2/4 50 0/4 0          |                         | Exon 8, codon 280: CGT→CAT   |
| X-ray       | 3/18 18 1/18 6          |                         | Exon 8, codon 283: GAG→AAG   |
| Plutonium   | 2/29 7 2/29 7           |                         |                               |

ND, no data. aLung tumors were analyzed for p53 protein with the anti-p53 antibody CM1. SSCP analysis was conducted for exons 4 to 9, and direct sequencing was used to determine the specific mutation. bDue to the small size of some neoplasms, not all were included in SSCP analysis.
Hydroxyl radicals, hydrogen atoms, or solvated electron species formed by the radiolysis of water and other adjacent molecules resulting from the passage of alpha particles emitted by plutonium can also cause cytosine deamination (23). Both transition and transversion mutations were detected in the K-ras gene in human adenocarcinomas associated with exposure to radon (24). The lack of a specific mutation profile in these human tumors may stem from the fact that most of these uranium miners were also smokers. Adenocarcinomas associated with tobacco use have a higher frequency for transversion than for transition mutations in the K-ras gene (1).

The difference between alpha particles and X-rays in targeting the K-ras gene may stem from the amount of energy deposited. Alpha particles deposit large amounts of energy in small volumes along their paths, while X-rays deposit relatively sparse amounts of energy. Estimation of DNA damage using p53 expression as a dosimeter indicates that to produce equivalent acute damage requires an absorbed dose of X-rays 10-fold higher than for alpha particles (25). Therefore, the energy deposited by plutonium alpha particles would be expected to generate a higher concentration of DNA damage than that deposited by X-rays.

The low frequency of K-ras mutations found in the diesel exhaust- and carbon black-induced carcinomas may result from the lack of participation of mutagenic organics in the induction of neoplasia by diesel exhaust. This conclusion is supported by a recent report showing that DNA adducts specific to polycyclic aromatic hydrocarbons are not increased in rats chronically exposed to diesel exhaust (26). The fact that K-ras mutations are also a rare and late event in rat lung carcinogenesis induced by the nongenotoxic carcinogen, beryllium metal, provides further support for the hypothesis that diesel exhaust acts through a nongenotoxic mechanism. However, the K-ras gene in the rat is not always a target for genotoxic carcinogens. For example, despite the dose-related correlation of carcinogen-specific adducts to the induction of rat lung neoplasms by the genotoxic carcinogen NNK (11), the expected mutations in the K-ras gene were not found in these neoplasms.

The lack of ras mutations in rat lung tumors induced by genotoxic exposures is not necessarily an anomaly when compared to human lung carcinogenesis. Activation of the K-ras gene is detected in <40% of human adenocarcinomas but rarely in squamous cell carcinomas. This histologic preference for mutation of the K-ras gene was not observed in rat lung tumors, a difference that may stem from the progenitor cells responsible for these two tumor subtypes in rat and human lung cancers (11, 27). Other oncogenes must also be involved in the development of non-small-cell lung cancers. DNA from cells transformed in vitro by X-rays (28, 29) or in vivo by NNK (19) could transmit their malignant phenotype to NIH 3T3 cells; however, the oncogenes responsible were not members of the ras gene family. Identification of these non-ras transforming genes would be invaluable for unraveling some of the mechanisms underlying cancer induction by genotoxic and nongenotoxic exposures.

Results summarized in this review indicate that the p53 gene may be involved in the development of some rat squamous cell carcinomas, while alterations in this gene have not been detected in adenocarcinomas. Consistent with these findings, several recent reports indicate that alterations in p53 are infrequently found in primary rat neoplasms regardless of tissue or etiology (30–37), with the exception of squamous cell carcinomas (38, 39). Mutations in this gene are also infrequent in murine lung tumors (40–42). While immunohistochemical staining revealed evidence of p53 inactivation in 14 squamous cell carcinomas, both SSCP analysis and direct sequencing detected mutations in only 3 of 13 immunoreactive samples that were examined. Given the extent and distribution of immunoreactivity in the majority of these tumors and the sensitivity of SSCP (43) and direct sequencing, it is unlikely that mutant alleles present would not have been detected. The p53 protein immunoreactivity in these neoplasms may be due to mutations outside exons 4 through 9, although this is unlikely because such mutations have rarely been associated with protein stabilization and occur in <5% of human neoplasms with p53 mutations (44).

Alternatively, p53 immunoreactivity may be due either to stabilization by other gene products or the disruption of protein degradation. Stabilization of p53 by another protein may correlate with functional inactivation, analogous to its inactivation and extended half-life when bound by the SV40 large-T antigen. Precedent for this finding exists in several other reports in which enhanced detection of p53 by immunohistochemistry occurs in
the absence of mutations within the conserved region (33, 44, 45). One known method of inactivating wild-type p53 is the overexpression of the MDM2 gene product, which may stabilize the p53 protein to detectable levels. Immunohistochemical staining of lung tumors induced by diesel exhaust, carbon black, or X-rays detected overexpression of MDM2 in only one squamous cell carcinomas induced by X-ray exposure (14,18). This tumor also stained positive for p53. The MDM2 gene was not amplified in the immunoactive X-ray-induced tumor, suggesting that the mechanism underlying this overexpression could stem from enhanced translation as described by Landers et al. (46).

It is clear that while mutation of the p53 tumor suppressor occurs in 37 and 65% of human pulmonary adenocarcinomas and squamous cell carcinomas, respectively, many cancers develop independently of this tumor suppressor gene. The recent identification of the p16INK4a gene at chromosome 9p21 (47) and other genes being identified at chromosomes 3p14, 3p21, and 3p25 (48) should provide additional candidate genes for analysis in rat lung tumors.

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