p53 Mutations in Formaldehyde-induced Nasal Squamous Cell Carcinomas in Rats

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Abstract

Formaldehyde induces squamous cell carcinomas in the nasal passages of rats following chronic inhalation exposure at concentrations of ≥10 ppm. We have examined the complementary DNA of the tumor suppressor gene p53 from 11 primary formaldehyde-induced tumors for mutation using DNA sequence analysis. A polymerase chain reaction-amplified fragment of the rat p53 complementary DNA containing the evolutionarily conserved regions II–V was directly sequenced from each tumor. Point mutations in the p53 complementary DNA sequence were found in 5 of 11 of the tumors analyzed. These data demonstrate p53 point mutations in formaldehyde-induced squamous cell carcinomas and indicate a common alteration in certain rat and human squamous cell carcinomas of the respiratory tract.

Introduction

Mutation of the p53 gene is the most common genetic alteration found in human tumors and occurs in a wide variety of human cancers (1–3). In addition to point mutations, a variety of genetic alterations including deletions, loss of allelic heterozygosity, and rearrangements of the p53 gene have been detected in human tumors. Studies on the spectrum of p53 mutations in human cancers have been of particular interest (2). Since endogenous and exogenous mutagens produce specific kinds of mutations, the spectrum of mutations at p53 may provide insights into the etiology of specific cancers. Mutations of the p53 gene in human cancers occur predominantly at evolutionarily conserved amino acid residues, with various tumor types displaying differences in the spectrum of p53 base substitution mutations (2, 3).

Formaldehyde is a major commodity chemical that induces a concentration-dependent increase in nasal cancer (SCC)2 in rats (4). The concentration-dependent increase in formaldehyde-induced tumors parallels the formation of DNA-protein cross-links, inflammatory responses in the nasal epithelium, and sustained increases in nasal epithelial cell proliferation (5–8). Formaldehyde is genotoxic for several end points, including gene mutation, chromosomal aberrations, and cell transformation (9–11). A higher frequency of micronucleated cells has been observed in the nasal mucosa of workers in a plywood factory with formaldehyde exposures relative to nonexposed controls (12). There have been no epidemiological studies offering convincing evidence of a correlation between exposure to formaldehyde and an increased incidence of human respiratory tract cancer (13).

Although much progress has been made on the analysis of human tumors for p53 alterations, studies in rodents, especially rats, are lacking. To examine the potential role of p53 gene mutations in rat tumorigenesis, we have analyzed the sequence of p53 cDNA in formaldehyde-induced rat tumors.

Materials and Methods

Squamous Cell Carcinomas. Tumor tissue was harvested from the nasal passages of F344 rats chronically exposed to 15 ppm formaldehyde gas for up to 2 years. Details of the formaldehyde generation system, chamber analysis, the source of the rats, and their housing and husbandry have been reported previously (6, 7). Tissues for the present study were collected from rats exhibiting clinical signs consistent with the presence of a nasal neoplasm (14). The nasal passages were dissected under sterile conditions, and fragments of tumor tissue were rapidly frozen in liquid nitrogen and stored at −70°C. Fragments of each tumor were embedded in paraffin, and 5-μm sections were cut, stained with hematoxylin and eosin, and examined by light microscopy to determine the tumor type. All tumors that had mutations in p53 had been examined and diagnosed by histopathology as SCC. The tumor samples without p53 mutations had been examined and diagnosed by histopathology as SCCs (FATu 15, 16, 37) and one poorly differentiated carcinoma (FATu 45). Two tumor samples (FATu 27, 43) did not have sufficient material for a confirmation of tumor type; however, based on previous studies they are likely to be SCC (4). The tumors analyzed in the present study are a subset of 29 tumors that had sufficient material available for RNA isolation.

Synthetic Oligonucleotide Primers. Oligomers were synthesized using a DNA synthesizer (model 381A; Applied Biosystems, Inc., Foster City, CA) and purified using oligonucleotide purification cartridges (Applied Biosystems, Inc.). The rat p53 cDNA sequence was used for the design of all oligomer primers (15). The A of the p53 start codon AUG was designated as base 1. The Oligo Primer Analysis software program (Version 3.4; National Biosciences, Hamel, MN) was used as an aid for the identification of potential oligomers for PCR amplification and DNA sequencing. The sequences of the synthetic oligomers used in this study are shown in Table 1.

RNA Isolation, Reverse Transcriptase cDNA Synthesis, and PCR Amplification. RNA was isolated from quick-frozen tumors pulverized under liquid N2, with guanidium thiocyanate, using the CsCl method (16). Rat p53 cDNA was synthesized using the RNA PCR kit from Perkin-Elmer Cetus (Norwalk, CT). Random hexamer primers were used to prime the cDNA synthesis reaction using approximately 1 μg of RNA. The cDNA synthesis reaction mixture was incubated at room temperature for 10 min and then incubated in a DNA thermal cycler (Perkin Elmer) at 42°C for 15 min, 99°C for 5 min, and 4°C for 5 min. The cDNA reaction mixture was added directly to a PCR mix containing 50 pmol each of two rat p53-specific primers (Table 1) that anneal upstream from the start codon (p53-1) and downstream from the stop codon (p53-4). The entire coding region of p53 cDNA was PCR-amplified in a DNA thermal cycler (Perkin Elmer) with the following parameters: 95°C for 1 min and 60°C for 2 min for 35 cycles, with a final extension step at 60°C for 7 min.

After examination of one-half of the PCR product on a 1% agarose gel, the remainder of the product was diluted 1:1000 and used as a template for a "second round" PCR to provide sufficient template for DNA sequencing. The strategy for the production of the second-round PCR products is illustrated in Fig. 1a. Appropriately paired primers (50 pmol of each primer) were used to produce fragment D. Standard PCR reaction buffer (Perkin Elmer) was used for this PCR amplification using the following temperature cycling parameters: 95°C for 2 min followed by 35 cycles of 95°C for 1 min, 30 s, 60°C for 2 min, with a last...
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Table 1 Sequence and location of oligonucleotide primers used for the PCR amplification and DNA sequence analysis of rat p53 cDNA

<table>
<thead>
<tr>
<th>Primer No.</th>
<th>Sequence (5'→3')</th>
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<tr>
<td>p53-1</td>
<td>9TAGTGAGCGATACATTAGGTAGGGTA-70</td>
</tr>
<tr>
<td>p53-4</td>
<td>120GGCTATAAAATGGCAGAAGGGGAGG-1215</td>
</tr>
<tr>
<td>p53-6A</td>
<td>263CCTGGGCTTCTGATCTCCGCTCC-287</td>
</tr>
<tr>
<td>p53-7-9B</td>
<td>98CTTAAGGTAAGATATCTCTCATGAC-102</td>
</tr>
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</table>

Results

p53 cDNA Sequence Analysis of Formaldehyde-induced Tumors. RNA from formaldehyde-induced tumors was reverse transcribed, and the p53 cDNA was PCR-amplified to obtain template for DNA sequencing. The cDNA synthesis and PCR amplification of rat p53 produces a 1.3-kilobase fragment that was used as a template for the synthesis of a second 723-base pair PCR product (fragment D) that contains the highly conserved regions II–V (Fig. 1a). These highly conserved regions correspond to the sites where approximately 75% of all the p53 mutations identified in human tumors occur (1–3). The PCR fragment containing these regions was directly sequenced in its entirety for each tumor sample. Representative results from the fluorescent DNA sequencer for the direct sequencing of fragment D from two primary tumors with point mutations are shown in Fig. 1b. The reverse transcriptase cDNA synthesis, PCR amplification, and DNA sequencing of fragment D were performed twice for each tumor sample. Point mutations were observed by sequencing with at least two primers. We consistently observe the wild-type sequence using this method from control samples.

DNA sequencing of the p53 cDNA from the rat tumors examined showed point mutations in 5 of 11 of the tumors (Table 2). The five point mutations observed occurred at GC base pairs (4 of 5 were transversions) and were homozgyous changes (see Fig. 1b) as indicated by the presence of a single fluorescent peak at the site of mutation. Four of five mutations occurred in the highly conserved region, while the fifth mutation occurred at an evolutionarily conserved amino acid. All of the mutated p53 codons identified in the rat SCC have been described as mutated in a variety of human cancers; one of the codons involved (rat codon 271, human codon 273) is considered a p53 mutational “hot spot” for human cancers (1–3).

Discussion

Following inhalation exposure of rats to irritant concentrations of formaldehyde (>6 ppm) a complex series of events occurs, involving initial epithelial cell death in specific regions of the nose (17, 18), followed by reparative and adaptive changes that are accompanied by increases in regional epithelial cell replication rates (7) and an inflammatory response. If the formaldehyde exposures are continued at a sufficiently high concentration (10 or 15 ppm), a number of proliferative lesions are induced, including hyperplasia, metaplasia, and putative preneoplastic changes (6, 17). After 1 year of exposure, benign and malignant squamous epithelial tumors arise, with the majority of tumors occurring in specific regions of the nose (6, 7, 19).

In the present study, we utilized reverse transcriptase-PCR (20) and DNA sequence analysis to determine the cDNA sequence of p53 from formaldehyde-induced tumors. Direct sequencing of the p53 cDNA showed that 5 of 11 of the formaldehyde-induced rat nasal tumors examined have point mutations. The mutations present in the primary tumors occurred at GC base pairs. All of the mutated codons observed in the present study have been mutated in human cancers (3). The proportion of human respiratory tract cancers that exhibit p53 alterations is similar to that found in the present study; a higher proportion of p53 mutations has been found in non-small cell lung cancers of the squamous cell type (21–28). Although the identical mutations observed in the present study have been observed in non-small cell lung cancers, these mutations have also been observed in other human cancers in organs other than the respiratory tract (3). In contrast, no mutations of p53 were observed in 35 CD-1 mouse lung tumors (29). Since the lung tumors examined were of non-squamous cell origin, this may reflect differences in target cell population. Mutations of p53 have been observed in other mouse tumors including fibrosarcomas, skin tumors, and lymphomas (30–32). Mutation of p53 occurs in rat renal-mesenchymal, esophageal, and Zymbal gland tumors (33, 34).

Since the frequency of background nasal SCC in rats is very low (35), the induction by formaldehyde of nasal SCC is particularly significant. Formaldehyde is genotoxic, inducing chromosomal aberrations, deletions, and point mutations (10–12). The formation of formaldehyde DNA-protein cross-links has been detected at concentrations of ≥0.3 ppm (36), increases nonlinearly with concentration, and is currently regarded as a measure of formaldehyde dose at the site of tumor formation.
Fig. 1. A, strategy for the PCR amplification of rat p53 cDNA: generation of a PCR product (fragment D) for DNA sequence analysis. Arabic numerals, start and stop nucleotide base numbers; Roman numerals, sites of evolutionarily conserved regions. PCR primer pairs necessary to synthesize desired p53 cDNA PCR products are listed in Table I. B, automated fluorescent DNA sequencer histogram at region of p53 cDNA with point mutations for two tumor samples. The DNA sequence shown is in the antisense direction.

(37). How DNA-protein cross-links or other potential formaldehyde-induced DNA adducts contribute to the induction of genetic alterations by formaldehyde is uncertain.

As indicated above, the induction of nasal SCC by formaldehyde is also accompanied by cytotoxicity and inflammation followed by regenerative cell proliferation that is particularly pronounced at the sites of tumor formation. The inflammation and proliferation induced by formaldehyde may contribute to the induction of genetic alterations through a variety of mechanisms including the generation of reactive oxygen species, alterations in nucleotide pools, free radical formation, and clonal expansion with further mutation of genetically altered cells (38–40). How these and other factors may lead to the induction of point mutations in p53 and alterations in other tumor suppressor genes and/or oncogenes is clearly an area requiring further investigation.

The present study represents our initial investigations into the role of tumor suppressor gene alterations in formaldehyde-induced nasal SCC. Although a number of biochemical and pathological alterations accompany the induction of nasal SCC induced by formaldehyde, this study is the first description of genetic alterations in formaldehyde-induced SCC. Our results show that 5 of 11 of the rat nasal tumors analyzed had a point mutation in the coding region of p53. Since p53 mutation occurs in the development of many human SCCs, these data indicate that certain human and rat SCCs share a common step in development. The observation that the point mutations were apparently homozygous changes indicates that the other allele of p53 is not expressed, or it has been deleted, or that there has been a recombinational event rendering both alleles of the p53 gene homozygous for the point mutations observed.

Since rodents are used to evaluate and assess the human risk for the potential carcinogenicity of a wide variety of agents, it is essential to determine the relationship of genetic alterations observed in human cancers to the induction of tumors in rats exposed to carcinogens. These studies can establish biologically...
relevant mechanistic linkages of molecular events leading to tumors in rodents with defined exposures to carcinogens. A significant advantage of studies with rodents is that animals can be euthanized at specific time points following exposures to carcinogens so that the role of genetic alterations can be examined in early "preneoplastic" lesions and subsequent tumors. The present study demonstrates that p53 mutations occur in formaldehyde-induced nasal SCCs, indicating the need to further investigate the role of this and other tumor suppressor/ oncogene alterations in formaldehyde-induced SCC.

Acknowledgments

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References


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