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Benzo[*a*]pyrene-induced murine skin tumors exhibit frequent and characteristic G to T mutations in the p53 gene

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ABSTRACT **Human tobacco-related cancers exhibit a high frequency of G to T transversions in the mutation hot spot region of the p53 tumor suppressor gene, possibly the result of specific mutagens in tobacco smoke, most notably benzo[*a*]pyrene (B[*a*]P). No *in vivo* animal model of B[*a*]P-induced tumorigenesis has been used, however, to substantiate these molecular epidemiological data experimentally. Direct DNA sequence analysis of the hot spot region (exons 5–8 inclusive) of murine p53 was performed in 20 skin tumors induced by a complete carcinogenesis protocol with B[*a*]P. Sequence analyses revealed numerous heterozygous missense mutations in carcinomas, specifically in exons 7 and 8 of the p53 gene, and targeting exclusively guanine residues. Moreover, 70% (5/7) of the mutations characterized were G to T transversions. In contrast, direct DNA sequence analysis of 36 skin tumors induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) in either a complete carcinogenesis protocol or in a two-stage carcinogenesis protocol revealed a 30% frequency of heterozygous p53 mutations, with the majority of mutations found in carcinomas, but only a single G to T transversion (1/8). Thus, while mutation frequencies are similar, the pattern and type of p53 mutations in B[*a*]P-induced skin tumors differs significantly from the mutation spectra in DMBA-induced squamous neoplasias. These *in vivo* findings in B[*a*]P-induced tumors lend support to *in vitro* and molecular epidemiological evidence, suggesting that the p53 tumor suppressor gene may be a selective target of metabolically activated B[*a*]P species etiologically associated with human tobacco-related cancers.**

Alterations in dominant oncogenes and recessive tumor suppressor genes are believed to constitute critical events in the multistep process of tumor development (1). Mutations in the p53 tumor suppressor gene have been characterized in a wide variety of human tumors (see refs. 2 and 3) and appear to be one of the most frequent genetic alterations in human neoplasias (1, 2).

An increasing body of molecular epidemiological evidence suggests that regions of the p53 gene may be a selective target of environmental carcinogens etiologically associated with specific human cancers (4–19). In this context, a high frequency of G to T transversions in the mutation hot spot regions of the p53 gene (2, 3) have been identified in human hepatocellular carcinomas and strongly correlated with the mutagenic effects of dietary aflatoxin B1 exposure (4, 13–15). Similarly, UV-B-specific dipyrimidine G to T transitions in the p53 gene have been characterized in human squamous cell carcinomas of the skin (16, 17). Unique mutations in p53 have likewise been reported in radon-associated lung cancers (18) and in human epithelial cells exposed to nickel(II) *in vitro* (19), providing evidence for an important molecular target for the genotoxic effects of these agents. Of particular clinical and epidemiological interest are the observations of a high frequency of G to T transversions among p53 mutations in tobacco-related human neoplasias, including small-cell and non-small-cell lung cancers (5–8), esophageal carcinomas (10–12), and squamous cell carcinomas of the head and neck (9). In several of these studies, the presence of p53 mutations in tumors was strongly correlated with lifetime cigarette consumption (7) or a history of heavy smoking (9).

It has been suggested (4–12) that the frequent G to T transversion in the p53 gene in tobacco-related cancers may be associated with specific mutagens in tobacco smoke, most notably activated benzo[*a*]pyrene (B[*a*]P) species (4, 20, 21). *In vitro* studies with DNA polymerase fingerprint analysis have demonstrated that activated B[*a*]P and aflatoxin B1 species cause a high frequency of G to T transversions in the wild-type p53 gene (4), but, to date, no *in vivo* animal model of B[*a*]P-induced tumorigenesis has been used to study the frequency and pattern of p53 mutations in primary tumors.

We have previously investigated the role of p53 alterations in the development and progression of the two-stage mouse skin tumorigenesis model induced by 7,12-dimethylbenz-[*a*]anthracene (DMBA) and promoted with phorbol 12-myristate 13-acetate (PMA) (22). In this study, we examined the frequency and pattern of p53 mutations in murine papillomas and squamous cell carcinomas induced by a complete carcinogenesis protocol using B[*a*]P by immunohistochemical analyses of the p53 protein and direct DNA sequence analyses of exons 5–8 of the murine p53 gene. Similarly, p53 sequence analyses of 36 murine skin tumors induced by either two-stage or complete carcinogenesis protocols with DMBA were used for a comparative analysis with tumors induced by B[*a*]P.

MATERIALS AND METHODS

Induction of Skin Tumors. Tumors were induced in Sencar mice by topical application of 200 nmol of B[*a*]P or 20 nmol of DMBA once weekly for up to 52 weeks (23). Tumors and lung metastases were characterized histopathologically as described (24) following hematoxylin/eosin staining. Primary explant cultures of skin tumors were prepared as described (22). Papillomas harvested for analyses were subjected to 35–38 weeks of B[*a*]P treatment, while carcinomas analyzed were subjected to 50 weeks of carcinogen exposure. In the complete carcinogenesis protocol with DMBA, papillomas and carcinomas used in molecular analyses were obtained from mice subjected to 34–52 weeks of carcinogen exposure. The two-stage carcinogenesis protocol using DMBA as initiator has been described (22).

Immunohistochemical Analysis of Murine p53. Tumors were trimmed of surrounding connective tissue and 5-μm

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Abbreviations: B[*a*]P, benzo[*a*]pyrene; DMBA, 7,12-dimethylbenz-[*a*]anthracene; PMA, phorbol 12-myristate 13-acetate.

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frozen sections of each specimen were fixed in acetone for 15 min at 4°C. Analysis of the p53 protein was done with hybridoma supernatants of monoclonal antibodies pAb421 (25) and pAb248 (26) at a dilution of 1:20 by a conventional avidin-biotin immunoperoxidase technique (Vectastain; Vector Laboratories) and with diaminobenzidine (1 mg/ml in 0.02% hydrogen peroxide) used as a chromogenic agent as detailed (27).

PCR Amplification and Sequence Analysis of the Murine p53 Gene. Genomic DNA was obtained from a total of 8 papillomas and 12 squamous cell carcinomas induced by B[*a*]P, and 3 papillomas and 33 carcinomas induced by DMBA, 8 of which were reported elsewhere (22), as described (28). Samples were subjected to 35 cycles of PCR amplification using murine-specific intronic primers for exons 5–8 inclusive of the p53 gene as described (29). All PCR amplification primers were contained within intron sequences to avoid amplification of the murine p53 pseudogene (29). Each amplification cycle consisted of 94°C (1.17 min), 62°C (1 min), and 72°C (1.25 min) after an initial denaturation step (94°C for 5 min). After purification of PCR products by Sephadex G-25 filtration, direct sequence analysis of each exon 5–8 of murine p53 was done using T4 [γ-^{32}P]polynucleotide kinase-end-labeled sequencing primers as described by Goodrow *et al.* (29). Tumor genomic DNA/PCR products were resequenced using corresponding sense or antisense sequencing primers for each exon and/or a separate PCR product to verify specific mutations (22, 29).

RESULTS

The complete carcinogenesis protocol with B[*a*]P and DMBA resulted in production of both papillomas and carcinomas, with the latter varying in histopathological grade from well-differentiated squamous carcinomas (grade I) to anaplastic, spindle-type tumors (grade IV). Moreover, many of the observed papillomas were marked by cellular atypia and dysplasia.

Table 1. Immunohistochemical analyses of B[*a*]P-induced skin tumors with antibodies pAb248 and pAb421

Tumor category	% positive nuclear p53 reactivity
Papillomas (n = 13)	0
Squamous cell carcinomas (n = 13)	62

Papillomas were obtained from mice subjected to 35–38 weeks of B[*a*]P exposure. Squamous cell carcinomas, grades I–IV, were obtained from mice subjected to 50 weeks of B[*a*]P exposure.

Immunohistochemical analyses of the p53 protein in frozen sections of 13 papillomas and 13 carcinomas induced by B[*a*]P with antibodies pAb421 and pAb248 revealed the absence of nuclear reactivity for p53 in all papillomas but positive nuclear staining in 62% of the carcinomas (Table 1 and Fig. 1 *A* and *B*). Carcinomas exhibiting positive nuclear reactivity for p53 ranged from stages I to IV in histopathological grade. As depicted in Fig. 1 *A* and *B*, frozen sections of immunohistochemically positive and negative tumors with pAb248 reveal some background epithelial staining, especially in the superficial layers, while primary explant cultures did not exhibit this pattern (Fig. 1*C*).

To confirm and characterize suspect p53 mutations in the B[*a*]P-induced tumors, direct sequence analysis of exons 5–8 inclusive of the murine p53 gene was undertaken in PCR-amplified genomic DNA from 8 papillomas, and 12 primary B[*a*]P carcinomas, 5 of which were immunohistochemically positive for nuclear p53 reactivity, 4 of which demonstrated no nuclear p53 staining, and 3 of which were selected randomly without immunohistochemical verification. As shown in Table 2 and Fig. 2, a single heterozygous mutation in one allele of the murine p53 gene was identified in only 1 papilloma in exon 8, codon 276, while several mutations were confirmed in both immunohistochemically positive and neg-

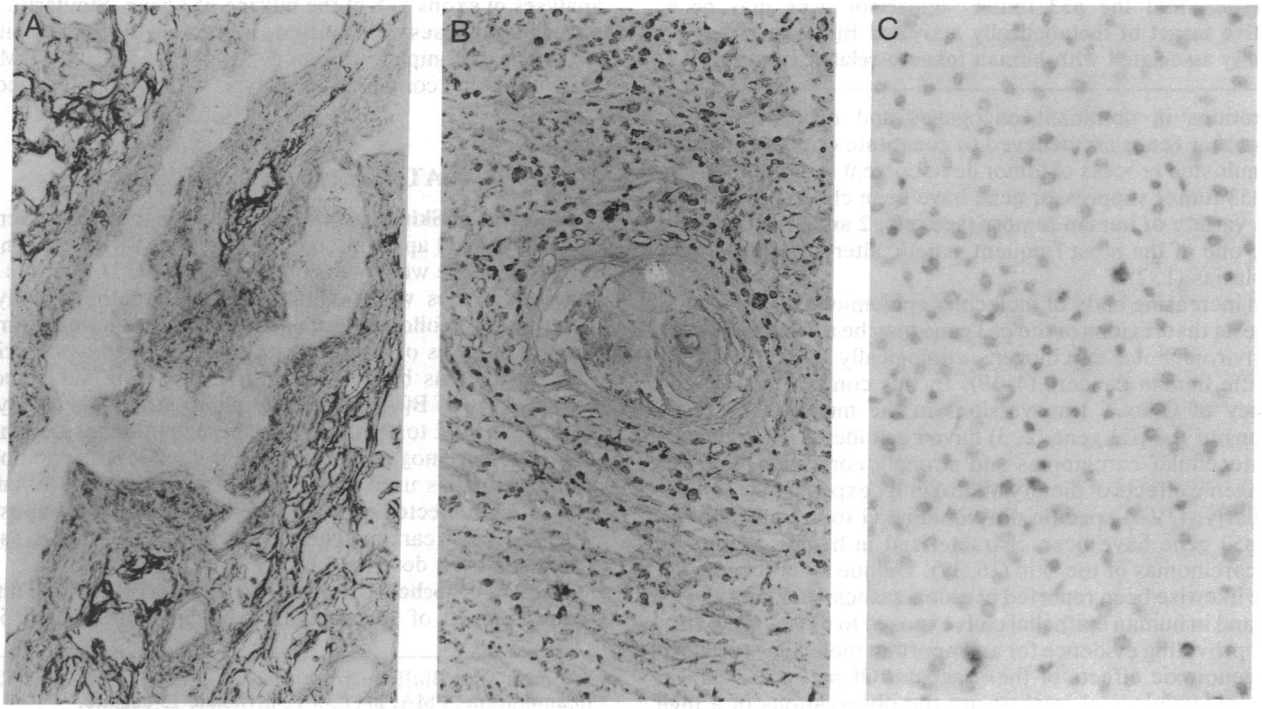

FIG. 1. Immunohistochemical analyses of murine p53 in B[*a*]P-induced skin tumors subjected to 50 weeks of B[*a*]P treatment. (*A*) Frozen section of an immunohistochemically negative atypical papilloma (pAb248). (*B*) Frozen section of an immunohistochemically positive squamous cell carcinoma, stage II (pAb248). (*C*) Primary short-term culture of an immunohistochemically positive squamous cell carcinoma (pAb248). Note the absence of background epithelial staining compared with frozen sections of primary tumors. Immunohistochemical analyses were confirmed with antibody pAb421. Methods are detailed in text and in ref. 27. (×360.)

Table 2. Summary of murine p53 analyses in B[*a*]P-induced and DMBA-induced papillomas and squamous cell carcinomas

Tumor	p53 immunohistochemistry	Histopathology grade	Exon/codon	Nucleotide change*	Amino acid change
Direct sequence analysis of exons 5–8 of murine p53 in B[*a*]P-induced skin tumors (n = 8 papillomas; n = 12 squamous cell carcinomas)					
91-3189	+	SCC, IV (50)	7/254	CTG → CTT	Leu → Leu
91-3114	+	SCC, II (50)	7/245	CGC → CTC	Arg → Leu
91-3198	+	SCC, II (50)	7/246	CCG → CCT	Pro → Pro
91-3198	+	SCC, II (50)	8/270	TGC → TTC	Cys → Phe
91-3277	−	SCC, II (50)	8/263	CGA → GAA	Gly → Glu
91-3277	−	SCC, II (50)	8/280	CGT → CTT	Arg → Leu
92-0565	−	Pap (50)	8/276	GGG → CCG	Gly → Pro
Direct sequence analysis of exons 5–8 of murine p53 in DMBA-induced skin tumors (n = 3 papillomas; n = 17 squamous cell carcinomas)					
86-1775		SCC I (52)	6/204	GAA → GAG	Glu → Glu
86-1800		SCC III (52)	5/151	GGG → AGG	Gly → Arg
86-1805		SCC I (52)	7/244	AAC → CAC	Asn → His
86-1805		SCC I (52)	8/299	GGG → GGT	Gly → Gly
1738		Atypical pap (46)	6/233	TAC → TAA	Tyr → Stop
86-1730		SCC III (46)	Intron 8	CGA → TGA	—
Direct sequence analysis of exons 5–8 of murine p53 in skin tumors induced with DMBA (two-stage carcinogenesis) (n = 16 squamous cell carcinomas)					
1		SCC IV	8/270	CGT → CAT	Arg → His
2		SCC I	6/219	CCA → CCG	Pro → Pro
3†		SCC II	7/245	CGC → CCC	Arg → Pro
4†		SCC II	8/263	GGA → GAA	Gly → Glu

Numbers in parentheses indicate weeks of exposure to carcinogen. SCC, squamous cell carcinoma; pap, papilloma.
*All mutations were heterozygous, affecting only one allele of murine p53.
†Two tumors from ref. 22.

ative carcinomas, predominantly in exons 7 and 8 of the p53 gene, giving rise to a mutation frequency of 33% in B[*a*]P-induced tumors. Two of the identified G to T transversions resulted in silent mutations at the amino acid level (Table 2). The data suggest that the absence of nuclear p53 immunoreactivity is not an absolute criteria for the presence of a wild-type p53 gene in this tumor model.

Most notably of the seven missense mutations in B[*a*]P-induced tumors, all confirmed on a second PCR product and/or by resequencing a given exon with a corresponding antisense primer, all mutations targeted guanine residues and 71% (5/7) were G to T transversions; the remaining two were G to A and G to C alterations. This pattern of G to T transversions is the predominant mutation induced by activated B[*a*]P diol epoxides in *in vitro* studies (4, 20, 21) and is suggestive of selective targeting of the p53 gene by these electrophilic species generated *in vivo* upon exposure to, and metabolism of, B[*a*]P (4).

To compare the frequency and types of p53 mutations in skin tumors induced by B[*a*]P with those of other carcinogens, sequence analyses were undertaken on 3 papillomas and 17 squamous cell carcinomas induced by a complete carcinogenesis protocol with DMBA. As shown in Table 2, a total of six heterozygous mutations were confirmed in DMBA-induced tumors, yielding a frequency similar to that observed in B[*a*]P-induced tumors. Moreover, the majority of mutations (5/6) were identified in squamous cell carcinomas; only a single atypical and dysplastic papilloma revealed a p53 mutation. Of the mutations identified in tumors induced by complete carcinogenesis with DMBA, three were missense mutations, two were phenotypically silent, and one was a nonsense mutation. Most notably, only a single G to T transversion was identified among these 20 tumors. Moreover, in a previous study of p53 alterations in DMBA/PMA-induced mouse skin tumors, 2 of 8 carcinomas revealed p53 mutations, none of which were G to T transversions (22). Sequence analyses of an additional 8 DMBA/PMA-induced skin tumors revealed a G to A transition in codon 270 and an A to G transition in codon 219 (Table 2). Thus, 4 of 16 tumors possessed p53 mutations, none of which were G to T transversions. Statistical analysis using the Fischer exact test of the frequency of G to T transversions in B[*a*]P-induced lesions versus those induced by DMBA revealed that the number of these transversions in relation to other types of mutations in B[*a*]P-induced tumors is significantly different (P = 0.017) from those seen in the DMBA-induced neoplasms.

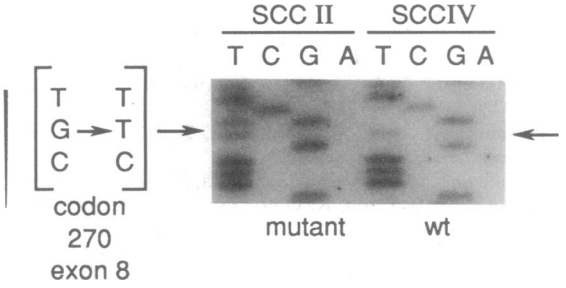

FIG. 2. Representative direct sequence analysis of murine p53 in B[*a*]P-induced skin tumors. PCR-amplified genomic DNA from primary tumors was subjected to direct sequencing with ^{32}P-end-labeled sequencing primers as detailed (22, 29). Mutated region is indicated by arrows. SCC, squamous cell carcinoma; wt, wild-type.

DISCUSSION

The results of the present study of p53 mutations in murine B[*a*]P-induced skin tumors reveal a 33% frequency of missense mutations predominantly G to T transversions in exons 7 and 8 of the p53 gene and a 62% frequency of immunohistochemically positive carcinomas. In contrast, papillomas induced by this protocol were immunohistochemically negative for p53 nuclear staining, and only a single p53 mutation was found by DNA sequence analysis. Mutations were not

identified in every immunohistochemically positive tumor by sequence analysis. The possibility that p53 mutations in these tumors reside outside of the highly conserved regions of the p53 gene or in promoter and enhancer regions of the gene, resulting in overexpression of the p53 protein, has been addressed by others (30). These *in vivo* findings lend support to *in vitro* analyses (4) and molecular epidemiological studies of human tobacco-related cancers (4–12), suggesting that activated B[*a*]P species may form adducts with predominantly guanine residues of the p53 gene and induce a high frequency of G to T transversions. Comparative analyses of the p53 mutation spectra in DMBA-induced skin tumors strengthens this supposition; while the frequency of mutations is similar, G to T transversions are rare in DMBA-induced and DMBA/PMA-induced (Table 2) skin tumor models of squamous cell neoplasias. This is supported by the fact that no tumor cell line derived from DMBA-induced tumors contains G to T transversions (22). Furthermore, Burns *et al.* (31) sequenced several skin tumors induced with DMBA and found 7 mutations, and only one G to T transversion. Similarly, G to T transversions in codon 12 of the Ha-*ras* oncogene predominate in B[*a*]P-induced mouse skin tumors (32), suggesting that a number of proliferation-related genes may be targeted by activated B[*a*]P species.

In addition to the B[*a*]P in tobacco smoke, *N*-nitrosamines may also exert mutagenic effects on the p53 gene (see ref. 11). Although the effects of *N*-nitrosamines in tobacco smoke have not been examined with respect to their mutagenic effects on the p53 gene, alkylating *N*-nitroso compounds (*N*-nitrosoethylurea and *N*-nitrosomethylurea) have been shown to induce a high frequency of G to A transition mutations in the rat p53 gene (33). Rat renal and esophageal tumors induced by these *N*-nitroso compounds exhibited selective targeting of G to A transitions predominantly in codons 204 and 213 in exon 6 of rat p53 (33).

B[*a*]P, a complete carcinogen, has both tumor-initiating and -promoting properties. The promoting effects of B[*a*]P in murine epidermal tumorigenesis has been studied (34) and shown to involve dose-dependent dermal and epidermal responses similar to those induced by tumor-promoting phorbol esters. Epidermal hyperplasia, an elevation of mitotic indices, and an increased density of dark and pyknotic cells have been observed after B[*a*]P exposure, along with elevated numbers of giant keratinocytes and keratinocyte mitotic arrest at the G_2/M boundary (34). Moreover, in both B[*a*]P-induced and DMBA/PMA-induced murine skin tumors, the initial tumor type is predominantly a benign papilloma (24, 35) although carcinomas may also arise directly from nonpapillomatous skin exposed to carcinogen (24).

Previous studies in our laboratory (22) and others (31) in the DMBA/PMA-induced model of murine skin tumorigenesis have demonstrated that alterations in the p53 gene are a later event in tumor development—i.e., found in squamous cell carcinomas and more advanced tumors, but absent in papillomas and hyperplastic lesions. Similarly, in the complete carcinogenesis protocols of B[*a*]P-induced and DMBA-induced skin tumorigenesis, p53 alterations appear to be a rare event in papillomas (35–50 weeks treatment) and predominate in carcinomas (50 weeks treatment). In contrast to our previous studies (22), however, there was no correlation in B[*a*]P-induced and DMBA-induced carcinomas between the frequency of p53 mutations and the histopathological grade of the carcinomas examined (Table 2). Similar findings have been reported in human non-small-cell lung cancers (6–8) in which predominantly G to T transversions in the p53 gene were not correlated with clinical stage, tumor size, or nodal metastatic behavior but were of a higher frequency in tumors of a squamous cell histology.

While the data presented provide *in vivo* evidence to suggest that activated B[*a*]P species target the p53 gene, comparing animal models to human cancers must nonetheless be made cautiously. Species-specific differences in carcinogen metabolism and DNA repair capabilities must be considered in addition to the involvement of other environmental agents in the process of tumor development. This is best exemplified by the low frequency of p53 mutations identified in aflatoxin B1-induced liver tumors in nonhuman primates (36), findings that are in marked contrast to *in vitro* (4) and human molecular epidemiological evidence (13–15) suggesting a selective targeting of the p53 gene by activated aflatoxin B1 species.

Studies to elucidate the molecular alterations that accompany tumor promotion and progression in several models of chemically induced murine skin tumorigenesis remain to be done. It remains to be explained how initial exposure of mouse skin to a carcinogen results in detectable p53 mutations predominantly in carcinomas and not in benign lesions, or only at a low frequency in the latter. Carcinomas bearing p53 mutations may arise directly from carcinogen-initiated nonpapillomatous skin independent of intermediate papilloma formation. Alternatively, the possibility exists that carcinogen-initiated cells possessing p53 mutations constitute a minority subpopulation that is expanded only after additional genetic and molecular alterations that accompany skin tumor promotion and progression to a more malignant carcinomatous state (35, 37) and, hence, are manifested phenotypically as later events.

In view of evidence for a role of wild-type mouse and human p53 in transcriptional regulation (38, 39), alterations in murine p53 may affect the transcription of genes important in skin tumor development. For example, cooperation between activated *ras* and *fos* oncogenes are believed to play a critical role in the malignant conversion of murine keratinocytes to the carcinomatous state (40). Expression of wild-type but not mutant murine or human p53 has been shown to repress the transcription of several important proliferation-related genes, including c-*fos* (41, 42), c-*jun* (42), and interleukin 6 (41). The influence of mutations in murine p53 on expression of these proliferation-related genes and the temporal sequence in which these alterations may occur in mouse skin tumor development have not been examined. These possibilities await clarification in order to understand the nature and sequence of events that accompany tumor development in murine skin carcinogenesis models of human epithelial neoplasia.

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