Dose-dependent mutation profile in the c-Ha-ras proto-oncogene of skin tumors in mice initiated with benzo[a]pyrene

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Female CD-1 mice were treated topically with a low (25–50 nmol) or high (800 nmol) dose of benzo[a]pyrene (BP) or acetone vehicle, followed by 5 nmol 12-O-tetradecanoylphorbol 13-acetate (TPA) twice a week for 26 weeks. Selective UV radiation fractionation followed by PCR methods were used to analyze histologically defined subsets of cells (~100–200 cells) on formalin-fixed, paraffin-embedded and H&E stained microscope sections. DNA samples from normal-appearing, hyperplastic or tumor regions from the skin of animals from each treatment group were isolated and amplified by PCR with c-Ha-ras-specific primers. Single-strand conformation polymorphism (SSCP) analyses were performed on both exon 1 and 2 products from each sample. DNA extracted from each aberrant band of SSCP analyses was amplified by PCR for further sequence analysis. The data indicate that c-Ha-ras mutations can be detected in normal-looking and hyperplastic epidermal cells as well as in tumor cells obtained from mice initiated with BP and promoted with TPA. The frequencies of c-Ha-ras mutations for normal-looking, hyperplastic and tumor samples were 3/20 (15%), 8/17 (47%) and 58/68 (85%), respectively, for the low dose group and 8/18 (44%), 10/20 (50%) and 64/86 (74%), respectively, for the high dose group. These observations indicate that there were no dose dependencies in the mutation frequencies for normal-looking, hyperplastic and tumor samples. For combined high dose and low dose samples, differences in mutation frequencies of the c-Ha-ras gene between the normal-looking, hyperplastic and tumor samples were highly significant (P < 0.0001, Fisher’s exact test). All mutations detected were located at codons 12, 13 and 61 of the c-Ha-ras gene. With the numbers in parentheses indicating the nucleotide position in the coding sequence of the c-Ha-ras proto-oncogene, the distributions of mutations for G→A (35), G→T (35), G→C (37), G→T (38), C→A (181), A→T (182) and A→G (182) in the low dose tumors were 5, 2, 11, 74, 0, 7 and 2%, respectively, and the distribution of mutations in tumors from animals treated with a high dose of BP were 3, 7, 13, 61, 15, 1 and 0%, respectively. Differences in the global mutation spectra (site and kind of all mutations) for the c-Ha-ras gene between the high and low dose group tumors were statistically significant (P < 0.004, Fisher’s exact test) and the major difference between these two groups was C→A (181) base substitutions. In summary, our data indicate that: (i) 79% of the BP/TPA skin tumors in CD-1 mice had c-Ha-ras mutations for the combined data for high dose and low dose tumors; (ii) the major mutations detected in BP/TPA skin tumors were G→T transversions; (iii) the global mutation profile in the c-Ha-ras proto-oncogene in skin tumors obtained after initiation with a low dose of BP was different from that obtained after initiation with a high dose of BP.

Introduction

Although most mutagenesis and carcinogenesis studies are done with high doses of mutagens/carcinogens, human exposures are usually very low. Because of concerns about the significance or relevance for humans of studies on the mutational profiles of high, cytotoxic concentrations of chemicals, we and others have initiated a series of studies on the mutational profile of low, non-cytotoxic concentrations of environmental carcinogens or their ultimate carcinogenic metabolites utilizing an endogenous gene in Chinese hamster V-79 cells (1–3) and human lymphoblastoid AH1-1 cells (4). In earlier studies, we demonstrated that (+)-(7R,8S,9S,10R)-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE), an ultimate carcinogenic bay region diol epoxide derived from benzo[a]pyrene (BP), and (–)-(1R,2S,3S,4R)-3,4-dihydroxy-1,2-epoxy-1,2,3,4-tetrahydrobenzo[c]phenanthrene (B[c]PhDE), a potent ultimate mutagenic/carcinogenic fjord region diol epoxide derived from benzo[c]phenanthrene, induced dose-dependent mutational profiles in the hypoxanthine (guanine) phosphoribosyltransferase gene (hpri) of Chinese hamster V-79 cells (1–3). As the dose of each diol epoxide decreased, the proportion of base substitutions targeted at AT base pairs increased significantly. The two ultimate carcinogens have their benzylic hydroxyl group in trans to their epoxide oxygen and have the same absolute configuration at the oxygen-bearing carbons for all of their oxygen substituents. We have now evaluated the possibility that treatment of mice with a high dose of BP results in tumors with a different mutational profile than tumors from mice treated with a low dose of BP. In the present study, we have compared mutations in the c-Ha-ras proto-oncogene of mouse skin tumors initiated with a high or a low topical dose of BP followed by promotion with 12-O-tetradecanoylphorbol 13-acetate (TPA). The results indicate differences in the global mutation spectra (site and
kind of all base substitutions) between the high and low dose groups. A preliminary description of this study was given earlier (5).

Materials and methods

Reagents

BP was obtained from the Sigma Chemical Co. (St Louis, MO). TPA was from the LC Laboratories (Woburn, MA). Proteinase K and Tag DNA polymerase were purchased from Boehringer Mannheim (Indianapolis, IN). The Sequenase kit and T4 polynucleotide kinase were from Amersham Life Science (Arlington Heights, IL). All four deoxyribonucleotide triphosphates were from Pharmacia Biotech (Piscataway, NJ). PCR and sequencing primers were prepared by Ransom Hill Bioscience Inc. (Ramona, CA). All enzymes were used according to the recommendations of the suppliers.

Tumor induction

Female CD-1 mice obtained from Charles River Mouse Farms (North Wilmington, MA) were maintained under a 12 h light/dark cycle at a constant temperature and humidity and they received water and food ad libitum. All mice had their backs shaved every 2 weeks and at least 24 h before topical dosing with 5 nmol of TPA. All animals were shaved at age 7 days. The experiment was terminated 1 week after the last cycle. S.-J.C. Wei et al.

Table 1. Primer sequences

| Primer | Sequence 5’→3’ | Positions
|--------|----------------|-----------
| PCR    | H1A: GCA GCC GCT GCT GAA GCT ATG A | E1–18→4   
|        | H1B: GTA GGC AGA GCT CAC CTC TAT A | E1+9→104  
|        | H2A: GTT GTT TTG CAG GAC TCC TA   | E2–12→119 
|        | H2B: GGC ATA GGT GGC TCA CCT GTA | E2+16→286 
| Sequencing | H1sa: CAA AGT GGT TCT GGA | 84→70  
|        | H1ts: ACA GAA TAC AAG C | 4→15  
|        | H2sa: CAG GTG GTC ATT GA | 334→347 
|        | H2ts: TGT TGT TGA TGG CA | 466→453 |

Numbers indicate the nucleotide positions in the coding region of the Ha-ras cDNA relative to the first base of the start codon. Intron positions are referred to the nearest exon (E), with + and – indicating downstream and upstream from the exon, respectively.

DNA sample preparation

Selective UV radiation fractionation (SURF) followed by PCR was used to analyze a specific subset of cells on stained microscopical sections (6). H&E stained tissue sections were diagnosed by a pathologist (Y.-R.L.). With guidance from the pathologist utilizing a microscope, small ink dots from a felt-tip pen were manually placed directly on microscopically pure cell sub-populations (~100–200 cells) of hyperplastic or tumor regions to protect these areas not directly connected to the tumor but separated from the tumor by areas of normal skin. Incubating these cells overnight in the lysis buffer containing 100 mM Tris–HCl (pH 8.0), 2 mM EDTA, 400 µM proteinase K was inactivated by boiling before the DNA was extracted from normal-looking, hyperplastic or tumor regions to protect these areas from subsequent UV inactivation. After UV irradiation of the slide at 310 nm for 3 h to inactivate DNA of the unprotected cells for ability to undergo PCR, the genomic DNA of the protected cells was extracted by incubating these cells overnight in the lysis buffer containing 100 mM Tris–HCl (pH 8.0), 2 mM EDTA, 400 µg/ml of proteinase K and 2.5% of NP-40 at 37°C. Proteinase K was inactivated by boiling before the DNA was amplified by PCR for mutation analysis. In order to examine the heterogeneity of a tumor, DNA samples from multiple regions of the same tumor were isolated and analyzed separately. All hyperplastic samples were collected from areas not directly connected to the tumor but separated from the tumor by some normal-looking tissues. Whenever possible, a region containing normal-looking tissue on the same section of each hyperplastic and tumor lesion was also analyzed for comparison. In this study, all DNA samples collected for Ha-ras mutation analysis were obtained from regions only containing epidermis.

Detection of c-Ha-ras mutations

DNA samples extracted from normal-looking, hyperplastic or tumor regions of mouse skin from each treatment group were amplified by hot start genomic DNA PCR (7) with primers specific for c-Ha-ras exon 1 (H1A and H1B; Table I) or exon 2 (H2A and H2B; Table I). In the PCR reaction for single-strand conformation polymorphism (SSCP) analysis, the 5’-end of one primer was labeled with [γ-32P]ATP by T4 polynucleotide kinase. Each PCR reaction consisted of 40 cycles of denaturation (30 s at 94°C), annealing (1 min at 60°C) and extension (30 s at 78°C) followed by a prolonged extension (8 min at 78°C) after the last cycle. SSCP analysis (8,9) was performed to screen for mutations in both exons 1 and 2 for each sample. The PCR products were denatured and analyzed on a 9% (for exon 1 products) or 7% (for exon 2 products) non-denaturing polyacrylamide gel. After exposure to X-ray film, DNA samples from each aberrant band were excised and amplified by PCR for further sequence analysis. Both strands of the PCR product were sequenced for the entire exon 1 and exon 2 by the dideoxy termination reaction with Sequenase (10). The nucleotide sequences of the sequencing primers for exons were combined with data obtained from mice treated with 50 nmol of BP , so that sufficient ‘low dose’ tumors were available for analysis. There were 30, 118 and 44 animals in the control, low and high dose groups, respectively. The number of skin tumors that developed was determined by gross examination and recorded bi-weekly throughout the study. The size and exact location of each tumor that developed in the low dose group were determined during the course of the study. The experiment was terminated 1 week after the last application of TPA. Dorsal skin was excised and fixed in 10% neutral buffered formalin. The fixed tissue was embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for histopathological and mutational analysis.

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|        | H2B: GGC ATA GGT GGC TCA CCT GTA | E2+16→286 
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|        | H2ts: TGT TGT TGA TGG CA | 466→453 |

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Results

Tumor multiplicity and incidence

Mice were initiated with a single topical application of either a low (25–50 nmol) or high (800 nmol) dose of BP in 200 µl acetone. Beginning 1 week after initiation, mice were promoted twice weekly with 5 nmol TPA. As shown in Figure 1, after 26 weeks of promotion, the low dose group had 1.04 tumors/mouse with an 84% tumor incidence of tumor-bearing mice; the high dose group had 7.77 tumors/mouse with an 84% tumor incidence. In the control group, only one tumor was detected among the 30 animals studied (0.03 tumors/mouse with a 3% incidence of tumor-bearing mice).

Histopathology of skin lesions

A total of 51 tumors from the low dose group and 70 tumors from the high dose group were characterized pathologically. Most of the tumors were papillomas. Carcinoma in situ was a rare event for skin tumors produced by the protocol used in this study; only one case was observed in the high dose group. There was one keratoacanthoma in the low dose group and six in the high dose group. While keratoacanthomas with mild dysplasia were detected in both high and low dose groups, keratoacanthomas with moderate dysplasia were only observed for the high dose group (four tumors). For early stage papillomas, there were three in the low dose group and only one in the high dose group. Although papillomas with severe dysplasia were only detected in the high dose group (four cases), papillomas with moderate dysplasia were observed for both high and low dose groups. In general, skin tumors induced in the high dose group were at a more advanced developmental stage than tumors in the low dose group.
Dose-dependent mutations of BP-induced tumors

SSCP analysis and characterization of c-Ha-ras mutations

Frequency of c-Ha-ras mutations in normal-appearing and hyperplastic epidermis and in epidermal skin tumors from mice treated with BP/TPA. For each tissue section, cells from normal-looking regions were analyzed along with hyperplastic and tumor regions. The numbers of independent normal-looking, hyperplastic and tumor samples examined were: three, one and one, respectively, for the control group; 20, 17 and 68, respectively, for the low dose group; 18, 20, and 86, respectively, for the high dose group. Figure 2 shows examples of the SSCP analyses for tissue sections with different single-strand conformations. DNA extracted from each aberrant band excised from the gel was amplified by PCR and subjected to sequence analysis. The kinds of mutations for the aberrant bands were characterized and are listed in Figure 2. Under our experimental conditions, we demonstrated that different kinds of mutations resulted in different SSCP profiles. In mice treated with a low dose of BP, c-Ha-ras mutations were observed in 3/20 (15%) of the normal-looking samples, 8/17 (47%) of the hyperplastic samples and 58/68 (85%) of the tumor samples (Table II). For the high dose group, c-Ha-ras mutations were observed in 8/18 (44%) of the normal-looking samples, 10/20 (50%) of the hyperplastic samples and 64/86 (74%) of the tumor samples (Table II). There were no mutations detected in the three normal-looking tissue samples and one hyperplastic sample from the control group (Table II). Our data indicate that c-Ha-ras mutations can be detected in normal-looking and hyperplastic epidermis as well as in tumors obtained from mice initiated with BP and promoted with TPA. Although there was a trend toward an increase in the proportion of the normal-looking samples with c-Ha-ras mutations as the dose of BP increased, this trend was not statistically significant (P = 0.074, Fisher’s exact test). Our results also demonstrate that there was no dose-dependent difference in mutation frequencies of the c-Ha-ras gene for hyperplastic samples or for tumor samples. Since there was no dose-dependent difference in mutation frequencies of the c-Ha-ras gene for normal-looking, hyperplastic or tumor samples, the data from the two groups were combined to further explore a possible correlation between the frequencies of c-Ha-ras mutations and the stages of neoplastic development. In tissue samples obtained from BP/TPA-treated animals, 29% of normal-looking samples, 49% of the hyperplastic tissue samples and 79% of the tumor samples had a c-Ha-ras mutation (Table II). Although the mutation frequencies of the c-Ha-ras gene were not significantly different between the normal-looking and hyperplastic samples (P = 0.10, Fisher’s exact test), the mutation frequencies of the c-Ha-ras gene were significantly different between the hyperplastic and tumor samples (P = 3.50×10^{-4}, Fisher’s exact test). The data indicate that mutation frequencies of the c-Ha-ras gene of the skin samples from mice that received BP/TPA treatment depend on the phenotype of the samples (a 2×3 table, P < 0.0001, Fisher’s exact test). The mutation frequency increased as the phenotype progressed from normal-looking to hyperplastic to tumor (P < 0.0001). All mutations detected in normal-appearing epidermis or hyperplastic epidermis from BP/TPA-treated animals were G→T transversions at the second G of codon 13 of the c-Ha-ras gene.

Dose-dependent differences in the profile of c-Ha-ras mutations in tumors from mice treated with BP. The kinds of base substitutions observed in the c-Ha-ras gene for different tumor samples in the control, low dose and high dose BP groups are listed in Table III. The distributions of mutations for G→A (35), G→T (35), G→C (37), G→T (38), C→A (181), A→T (182) and A→G (182) in the low dose group were 5, 2, 11, 74, 0, 7 and 2%, respectively, and the distributions of these mutations in the high dose group were 3, 7, 13, 61, 15, 1 and 0%, respectively. The numbers in parentheses indicate the nucleotide position in the coding sequence of the c-Ha-ras gene. The one tumor observed in control mice (acetone/TPA treatment) had an A→G (182) transition mutation. Although only the mutation C→A (181) was significantly different (P = 0.001, Fisher’s exact test), when individual base substitutions were compared between the two dose groups, differences in the global mutation spectra (site and kind of all substitution mutations, a 7×2 table) of the c-Ha-ras gene between the high and low dose group tumors were statistically significant (P < 0.004, Fisher’s exact test). The major differences observed between the high and low dose tumor groups were mutations at exon 2 of the c-Ha-ras gene (a 3×2 table, P < 0.001, Fisher’s exact test).

Discussion

The use of selective UV radiation fractionation followed by PCR allowed the specific and sensitive molecular analysis of genes in a small subset of histologically defined cells (6). By using this method we were able to analyze c-Ha-ras mutations.
from 100–200 cells of normal-looking, hyperplastic and tumor regions on a histological slide. Since a mutation in 3% of the cell population can be detected by PCR–SSCP analysis (12), as little as three to six mutant cells can be detected from a sample with 100–200 cells of normal-looking, hyperplastic and tumor regions included with the sample to show complete inactivation of unprotected region; Bk, blank region (no tissue); subscripts, different regions of the tissue section.

**Table II.** Frequency of c-Ha-ras mutations detected in normal-looking, hyperplastic and tumor samples obtained from mice initiated with BP and promoted with TPA

<table>
<thead>
<tr>
<th>Sample</th>
<th>H-ras phenotype</th>
<th>Number of samples (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control Low</td>
<td>High</td>
<td>Combined</td>
<td></td>
</tr>
<tr>
<td>Normal-looking</td>
<td>Wild-type</td>
<td>3 (100)</td>
<td>17^{ab}</td>
<td>10^{bc}</td>
<td>2^{cd}</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>0 (0)</td>
<td>3^{cd}</td>
<td>8^{cd}</td>
<td>11^{cd}</td>
</tr>
<tr>
<td>Hyperplastic</td>
<td>Wild-type</td>
<td>1 (100)</td>
<td>9^{bc}</td>
<td>10^{bc}</td>
<td>19^{cd}</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>0 (0)</td>
<td>8^{bc}</td>
<td>10^{bc}</td>
<td>19^{cd}</td>
</tr>
<tr>
<td>Tumor</td>
<td>Wild-type</td>
<td>1 (100)</td>
<td>10^{bc}</td>
<td>22^{bc}</td>
<td>32^{cd}</td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>1 (100)</td>
<td>58^{bc}</td>
<td>64^{bc}</td>
<td>12^{cd}</td>
</tr>
</tbody>
</table>

CD-1 mice were initiated with a single dose of either 25–50 (low dose) or 800 nmol (high dose) of BP and promoted twice weekly with 5 nmol of TPA for 26 weeks. Control animals received only acetone during the initiation treatment, followed by TPA promotion. DNA samples from normal-looking, hyperplastic and tumor regions from mouse skin were analyzed for c-Ha-ras mutations as detailed in Materials and methods. Statistical analysis was evaluated for entries with the same superscript.

**Table III.** Mutations in c-Ha-ras detected in tumor samples obtained from mice initiated with a high or low dose of BP

<table>
<thead>
<tr>
<th>Base substitution (nucleotide no.)</th>
<th>Amino acid change (codon no.)</th>
<th>Number of mutations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G→A (35)</td>
<td>Gly→Glu (12)</td>
<td>2^{bc} (3)</td>
</tr>
<tr>
<td>G→T (35)</td>
<td>Gly→Val (12)</td>
<td>5^{bc} (7)</td>
</tr>
<tr>
<td>G→C (37)</td>
<td>Gly→Arg (13)</td>
<td>10^{bc} (13)</td>
</tr>
<tr>
<td>G→T (38)</td>
<td>Gly→Val (13)</td>
<td>46^{bc} (61)</td>
</tr>
<tr>
<td>C→A (181)</td>
<td>Gln→Lys (61)</td>
<td>1^{bc} (1)</td>
</tr>
<tr>
<td>A→T (182)</td>
<td>Gly→Leu (61)</td>
<td>4^{bc} (7)</td>
</tr>
<tr>
<td>A→G (182)</td>
<td>Gln→Arg (61)</td>
<td>1^{bc} (2)</td>
</tr>
</tbody>
</table>

For each slide analyzed, an unprotected tissue control was included with the sample to show complete inactivation of
DNA of the unprotected region, which ensured that the results obtained were not jeopardized by contamination of unprotected cells.

c-Ha-ras mutations in normal-looking epidermis from mice treated with BP and TPA

The presence of an activated Ha-ras proto-oncogene in normal-looking skin from mice initiated with BP and promoted with TPA demonstrated that activation of the ras proto-oncogene may precede the onset of neoplasia (Table II). This observation also indicated that the presence of c-Ha-ras mutations in the epidermis was not sufficient to efficiently trigger neoplastic development. It is possible that additional factors are needed to cooperate with activated c-Ha-ras in order to trigger a neoplastic conversion. Several earlier studies suggest that in addition to activation of the ras proto-oncogene, papilloma formation required the cooperation of proliferative stimuli (13–16) or TGF-α-mediated Ha-Ras-independent pathways (17,18). Our results with normal-looking skin samples that possess an oncogene mutation after BP and TPA treatment are similar to earlier data on Ha-ras mutations that were observed in histologically normal-looking tissue from the 7,12-dimethylbenz[a]anthracene (DMBA)- or urethane-initiated epidermis of mice (19) and nitrosomethylurea-treated mammary glands of newborn Sprague–Dawley rats (15). It is possible that our normal-looking skin samples with an active ras oncogene lacked additional factors needed for tumor development. Alternatively, Ha-ras mutations may not have an important role in skin tumor formation. The observation of the same Ha-ras mutation in certain normal-looking tissue samples as in the adjacent tumors was also reported earlier for some human tumors (20–22).

Tumors with no mutations in the c-Ha-ras gene

Twenty-one percent of the skin tumors analyzed had no mutations in the c-Ha-ras gene (Table II), indicating that Ha-ras activation was not required for skin tumor development. Multiple signaling pathways (Ha-Ras-dependent and Ha-Ras-independent) might be responsible for skin tumorigenesis in mice. Previous findings that papillomas can be generated by promotion of TGF-α transgenic mice without mutations in the c-Ha-ras gene also suggest that a Ha-Ras-independent signaling pathway was involved in skin tumorigenesis in these mice (23).

Heterogeneity of mutations in tumor samples

Although 149 of 154 tumor samples analyzed in the present study showed homogeneity in the genotype of the c-Ha-ras proto-oncogene and indicated a clonal origin of these tumors, five tumors were heterogeneous for c-Ha-ras base substitutions. We detected two different single base substitutions in c-Ha-ras sequences in different regions of the same tumor for three low dose and two high dose tumors. Two of the low dose tumors were large (6–7 mm diameter) and they appeared relatively early (weeks 16 and 18 after BP treatment, respectively). Since we followed the pattern of tumor growth for animals treated with a low dose of BP, it was unlikely that these two tumors resulted from the fusion of two individual tumors. Our finding that two different kinds of c-Ha-ras single base substitutions existed in the same tumor suggests the possibility that these tumors might have derived: (i) from a single cell in which each c-Ha-ras allele had a different single base substitution; (ii) from two separately initiated cells each with a different c-Ha-ras mutation. In recent studies, genetic heterogeneity was observed for human primary prostate tumors (24) and colorectal adenomas (25).

Frequencies of c-Ha-ras mutations in normal-looking and hyperplastic epidermis and in epidermal tumors

Mutation frequencies of the c-Ha-ras gene for skin samples from mice that received BP treatment were associated with the phenotype (normal-looking, hyperplastic or tumor) of the samples (Table II). The frequency of c-Ha-ras mutations increased from normal-looking samples to hyperplastic samples to neoplastic samples ($P < 0.0001$ for the combined dose groups, Fisher’s exact test). This observation suggests that c-Ha-ras activation might be an early event for many of the skin tumors. It is also possible that other factors cooperated with the activated Ha-ras oncogene and promoted the expansion of initiated cells as the cells advanced from normal to hyperplastic to grossly observable tumors.

Although we sequenced all of exons 1 and 2 of the c-Ha-ras gene for each sample, all mutations detected in BP/TPA-treated tissue samples were in codons 12, 13 and 61 (Table III). It is possible that for hyperplastic and tumor samples these mutation hotspots result from biological selection of the clonal expansion of cells with an activated Ha-ras gene. However, it is interesting to note that in normal-looking samples where no biological selection occurred, only G→T (38) transversions were detected (3/20 for the low dose group and 6/18 for the high dose group). This observation contradicts the concept of broad/multiple mutation targets for BPDE-induced mutations at hprt gene (2,26–28) and in shuttle vectors (29,30). It was postulated that the pausing of DNA polymerase at codons 12 and 13 of the c-Ha-ras gene due to the potential secondary structure in this region might contribute to the generation of clustered mutations at codons 12 and 13 (31). Possible mechanisms by which DNA conformation could enhance mutagenesis and account for hotspot formation might include: (i) the arrest of the DNA replication fork at hotspots, thereby increasing the probability of misincorporation; (ii) the unusual DNA conformation surrounding the hotspot, which could present a preferential site for the interaction of DNA with mutagens; (iii) the diminution of DNA repair in regions with unusual DNA conformation.

Kinds of mutations in the c-Ha-ras gene in skin samples from mice treated with BP and TPA

The most frequently detected mutation in tumors was G→T (38) transversion, with a frequency of 61% for the high dose group and 74% for the low dose group. We did not find any correlation between the kind of base substitutions and the pathological grade of the skin tumors. G→T (38) transversions were detected for papillomas with or without mild, moderate or severe dysplasia. It is interesting to note that none of the four early papillomas had a mutation in the c-Ha-ras gene. There was no correlation between the kind of base substitution and the growth rate of tumors. G→T (38) and G→C (37) transversions were detected for both large and small tumors.

The frequency and spectrum of Ha-ras mutations in 154 BP-initiated/TPA-promoted tumors in CD-1 mice from our study were similar to an earlier analysis of 10 papillomas from CD-1 mice by Colapietra et al. (32). In contrast to the results with CD-1 mice, most of the BP-initiated/TPA-promoted papillomas (77%, 10 of 13) from SENCAR mice did not have detectable mutations in c-Ha-ras codons 12, 13 or 61 (33). In our present study with CD-1 mice, there was only one tumor in 30 control animals treated with 5 nmol TPA twice weekly.
(3% tumor incidence and 0.03 tumors/mouse; Figure 1) and this tumor had an A→G (182) transition. In contrast to these results, the tumor incidence and multiplicity for SENCAR mice receiving TPA treatment alone were 13% and 0.13 tumors/mouse, respectively (33), and A→T (182) transversions were the major mutation detected in these tumors (33,34). These observations suggest that strain variance plays an important role in the frequency of mutations and possibly in the spectra of Ha-ras mutations in papillomas from animals treated with BP/TPA or only with TPA.

The major hotspot for c-Ha-ras mutations in tumors from BP-treated mice resembles a major hprt hotspot in V-79 cells treated with BPDE

BPDE is an ultimate carcinogen formed during mammalian metabolism of BP (35–40). Earlier studies demonstrated that BPDE and its dihydrodiol precursor were the major ultimate and proximate carcinogenic and mutagenic metabolites of BP (41,42). In these studies, BPDE was a potent lung carcinogen when injected i.p. into newborn mice and a potent skin carcinogen when applied topically to adult mice; the other three stereoisomers of the BP bay region diol epoxides had little or no carcinogenic activity (41,42). The finding that GC→TA transversions are the predominant mutations observed in BP-initiated tumors (83 and 76% for the high and the low dose groups, respectively) is in accord with cell culture studies, indicating that GC→TA transversions are the major kinds of mutation induced by BPDE (1,2,43,44). When the spectra of Ha-ras mutations from BP/TPA-induced tumors are compared with the spectra of hprt mutations induced by BPDE in our earlier study (2), it is interesting to note that both the target sequence (AQQG and the kind of mutation observed (G→T transversion at the underlined G) for the hotspot at G38 in the c-Ha-ras gene of mouse tumors are the same as for the hotspot at G47 of the hprt gene in V-79 cells (2). The observation that the mutational spectrum of BP-initiated/TPA-promoted tumors has the signature of BPDE-induced mutations strongly suggests that BPDE is responsible for the transforming mutations associated with papilloma formation in mouse skin initiated by BP and promoted with TPA. An alternative pathway for tumor formation in mouse skin was proposed to involve the unrepaired apurinic sites generated by loss of unstable hydrocarbon–DNA adducts for various polycyclic aromatic hydrocarbons (45,46). Although Cavalieri and co-workers reported that the major DNA adducts formed in mouse skin from BP, DMBA and dibenz[a,l]pyrene (DB[a,l]P) were unstable adducts which resulted in apurinic sites (47–50), Melendez-Colon et al. only detected stable adducts formed by DB[a,l]P activated in MCF-7 cells and no apurinic sites were detected from the DNA isolated (51). This latter study strongly suggests that DB[a,l]P exerts its biological effects through the formation of stable DNA adducts rather than by the induction of unstable adducts that lead to apurinic sites (51).

Effect of the dose of BP on the profile of base substitution mutations in the c-Ha-ras proto-oncogene in tumors

C→A (181) transversions in the c-Ha-ras proto-oncogene were detected in tumors from mice treated with a high dose of BP but not in tumors from animals treated with a low dose of BP (Table III). For the high dose group, mutations were almost entirely targeted at GC base pairs (99%). Although the number of tumors with an A mutation was small, the proportion of A→T (182) transversions in the c-Ha-ras proto-oncogene of tumors was increased 7-fold when tumors from animals treated with a low dose of BP were compared with tumors from animals treated with a high dose of BP. These observations are consistent with our previous findings, indicating that treatment of Chinese hamster V-79 cells with BPDE caused mutations predominantly at GC base pairs and that the proportion of mutations targeted at AT base pairs increased severalfold as the dose of BPDE was decreased (1,2,52). When the global mutation spectra (site and kind of all base substitutions) were compared for tumors obtained from mice treated with a high or low dose of BP, the differences were statistically significant (P < 0.004, Fisher’s exact test). Our data indicate a dose-dependent difference in the global mutational profile in the c-Ha-ras gene of BP-initiated/TPA-promoted tumors. Possible explanations for this dose dependence in the mutational profile might be: (i) a dose-dependent difference in the metabolic activation of BP resulting in a different profile of metabolites; (ii) a dose-dependent difference in diol epoxide binding to specific target bases on the DNA; (iii) a dose-dependent difference in the repair of DNA adducts from specific nucleotides at the target gene; (iv) a dose-dependent difference in the modulation of non-DNA targets resulting in different activities of DNA polymerases and DNA repair enzymes; (v) a dose-dependent difference in the proliferation and/or apoptosis of cells bearing specific adducts/mutations. Further experiments are required to evaluate these possibilities. Although no dose dependence in the ratio of G to A adducts in total cellular DNA was observed after exposing V-79 cells to BPDE (53), the ratio of A/G adducts in the hprt gene could be altered by the dose of BPDE. Using DNA repair-deficient VH-1 cells (a variant of Chinese hamster V-79 cells), we have demonstrated a lack of BPDE-induced dose-dependent mutational profile at the hprt gene (54). These results suggest that intact DNA repair activities are required for BPDE-induced dose-dependent mutational profiles.

There have been a few reports on dose-dependent mutational profiles in chemically induced tumors. When skin tumors induced by a racemic fjord region benzo[a]phenanthrene diol-epoxide-2 (ultimate carcinogen) in CD-1 mice were analyzed for mutations at codon 61 of the c-Ha-ras gene, a dose-dependent difference in the frequency of CAA→CTA mutations was observed. Increased mutations at AT base pairs were observed with decreasing dose, but mutations in other c-Ha-ras sequences were not examined (55). These observations are in agreement with our studies indicating that decreasing the concentration of B[cl]PhDE increased the proportion of base substitution mutations at AT base pairs and decreased the proportion of mutations at GC base pairs in the hprt gene of Chinese hamster V-79 cells (3). In other studies, mutations in the Ki-ras gene were examined from lung tumors induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in A/J mice and a dose-dependent difference in the frequency of GGT→GAT mutations at codon 12 was reported (56). However, this conclusion was not very strong since it was based on the analysis of 46 low dose tumors (56) that were compared with mutation profile data from 10 high dose tumors obtained in a different laboratory (57). It is of interest that the dosing procedure has also been reported to affect the pattern of ras gene mutations in liver tumors induced by 4-aminobenzene, N-hydroxy-2-acetylaminofluorene and N-nitrosodiethylamine in CD-1 mice. For each of these carcinogens, the multiple low dose tumor group (versus a single high dose group) had fewer Ki-ras and N-ras mutations and more Ha-ras codon 61 (C→A) mutations (58). Our present findings and the reports of others


