SENCAR Mouse Skin Papillomas Induced by 6-Methylbenzo[*a*]pyrene Carry H-*ras* Codon 52 ($CTA^{Leu} \rightarrow CCA^{Pro}$) and Codon 13 ($GGC^{Gly} \rightarrow GTC^{Val}$) Mutations

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Abstract: Organic smoke contains various methylated polycyclic aromatic hydrocarbon (PAH) carcinogens. Among these, 6-methylbenzo[*a*]pyrene (6-CH_3BP) is a moderate carcinogen. *In vitro*, it can form both stable and depurinating adducts. Of these, only two depurinating adducts [BP-6-CH_2-N7G and 6-CH_2BP-(1&3)-N7G] have been detected in mouse skin. We examined seven 6-CH_3BP-induced SENCAR mouse skin papillomas for the presence of oncogenic H-*ras* mutations. Two papillomas contained the codon 52 ($CTA^{Leu} \rightarrow CCA^{Pro}$) and a third contained the codon 13 ($GGC^{Gly} \rightarrow GTC^{Val}$) mutation. These results suggest that the H-*ras* codon 52 mutation is an oncogenic mutation.

Keywords: H-*ras* mutations, 6-CH_3BP, mouse, papilloma.

INTRODUCTION

Organic smoke, such as automobile exhaust, cigarette and forest fire, has long been known to contain various methylated polycyclic aromatic hydrocarbons [1-3]. Among these pollutants, methylated derivatives of benzo[*a*]pyrene (BP) may be important for cancer. Mono-methylated BP at positions 7, 8, 9 or 10 did not show significant carcinogenicity [4]. In contrast, 6-methyl BP (6-CH_3BP) (Fig. **1**) is mutagenic [5, 6], teratogenic [7] and carcinogenic, although it is a weaker carcinogen than BP [8-11]. In addition, 6-CH_3BP is produced by bioalkylation of BP with rat liver cytosol and S-adenosyl methionine, and therefore, has been suggested as a possible contributor in BP carcinogenesis [12, 13].

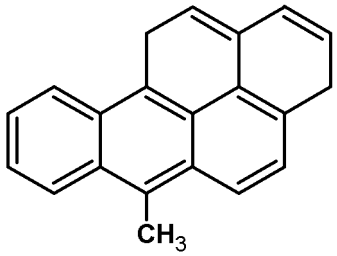

Fig. (1). Structure of 6-methylbenzo[*a*]pyrene.

To induce mutations, 6-CH_3BP needs to be metabolically activated by cytochrome P_{450}-mediated reactions [6, 14]. Activation of 6-CH_3BP by cytochrome P450 can generate a number of DNA-reactive metabolites, including a dihydrodiolepoxide and a radical cation [8, 13, 15]. The resulting stable DNA adducts have been analyzed by the ^{32}P-postlabeling assay, and the depurinating DNA adducts have been analyzed by HPLC. Comparative studies of DNA adduct formation *in vitro* and in mouse skin have identified the 6-CH_3BP radical cation as the major DNA-reactive metabolite in mouse skin [15-21]. The 6-CH_3BP radical cation reacts with DNA through its methyl carbon. *In vitro*, the radical cation forms both stable (BP-6-CH_2-N^2dG and BP-6-CH_2-N3dT) and depurinating adducts [BP-6-CH_2-N7G, 6-CH_2BP-(1&3)-N7G and BP-6-CH_2-N7A]. In mouse skin, however, only the depurinating Gua adducts have been positively identified [21].

No previous study has explored whether the 6-CH_3BP-DNA adducts are important for cancer. We have approached this question by examining H-*ras* mutations in 6-CH_3BP-induced papillomas and sought to make a correlation between DNA adducts and mutations.

MATERIALS AND METHODS

Chemicals

BP was purchased from the Chemical Carcinogen Repository of the National Cancer Institute and 6-CH_3BP synthesized in our laboratory as described previously [21]. *These PAH are hazardous and handled according to NIH guidelines* [22].

Papillomas

Tumors were induced by the initiation-promotion protocol. To obtain these tumors, 8-wk-old female SENCAR mice (National Cancer Institute-Frederick Cancer Center, Frederick, MD) were treated on the back with 160 or 400 nmol 6-CH_3BP in 100 μL acetone and, beginning the second week, promoted by twice weekly doses of 3 nmol 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) in 100 μL acetone, until the papilloma induction was complete (7-9 wks). The 160 nmol 6-CH_3BP treatment group yielded 5 papillomas in 30 mice and the 400 nmol 6-CH_3BP treatment group yielded 4 papillomas in 29 mice. All tumors were stored frozen at -80°C. Of these, seven papillomas were available for this study.

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Analysis of H-*ras* Mutations

Chromosomal DNA from the papillomas was isolated as previously described [23]. In the SENCAR mouse model, the papillomas contain clonally expanded, oncogenic H-*ras* mutations. For analyzing these mutations, a 550 bp-long segment spanning the exon 1-2 region was PCR amplified with primers MRF and MRR and the products directly sequenced forward and reverse primers as previously described [23]. Direct sequencing of PCR products can detect mutations that exist in at least 10% of the DNA molecules [24]. Since clonally expanded oncogenic H-*ras* mutations are typically present in 14-47% of cells in PAH-induced papillomas [25], direct sequencing of H-*ras* PCR products is an appropriate method for identifying these mutations. The PCR products were sequenced with primers described previously [23], and the reaction products were resolved in a Beckman/Coulter CEQ2000XL 8-capillary DNA sequencer (Genomics Core Research Facility, University of Nebraska, Lincoln). The clonal H-*ras* mutations in papillomas are heterozygous, and the sequencing electropherogram detects them as overlapped peaks corresponding to the mutant and wild-type bases.

RESULTS

We analyzed seven papillomas induced by 6-CH_3BP. One of these papillomas (14%) contained the codon 13 GGC^{Gly} to GTC^{Val} mutation, and another two (28%) contained a codon 52 CTA^{Leu} to CCA^{Pro} mutation (Fig. **2**). The remaining four papillomas did not contain any clonal H-*ras* mutations in the exon 1-2 region.

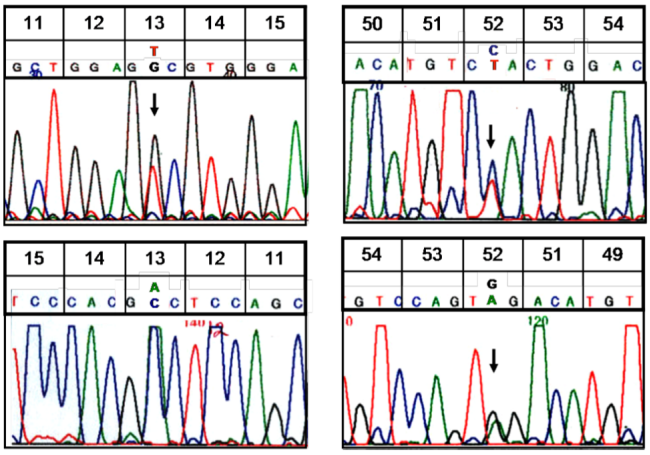

Fig. (2). Direct sequencing of H-*ras* PCR products from 6-CH_3BP-induced papillomas. Sequencing reactions were conducted with both forward (top) and reverse (bottom) primers as described previously [23]. The left panel shows typical results obtained for the codon 13 region and the right panel shows those for the codon 52 region. Also shown are the codon numbers and wild-type sequences, along with the mutations (above).

Codon 52 is located in a highly conserved *ras* region [26]. Deletion analyses have identified this region to be essential for transforming activity [27]. We conducted a molecular modeling study (Sybyl v 6.0, Tripos Associates, Inc., St Louis, MO) using co-ordinates from the Protein Data Bank (Brookhaven National Laboratory [28, 29]) to examine whether the codon 52 Leu^{52} to Pro mutation induces a gross structural change (Fig. **3**). The analysis predicted that codon 52 is located in the expanded region of a β-sheet (spanning from Glu^{49} to Thr^{58}), and the Leu^{52} to Pro substitution would not induce a major change in the three-dimensional structure of the ras protein [30].

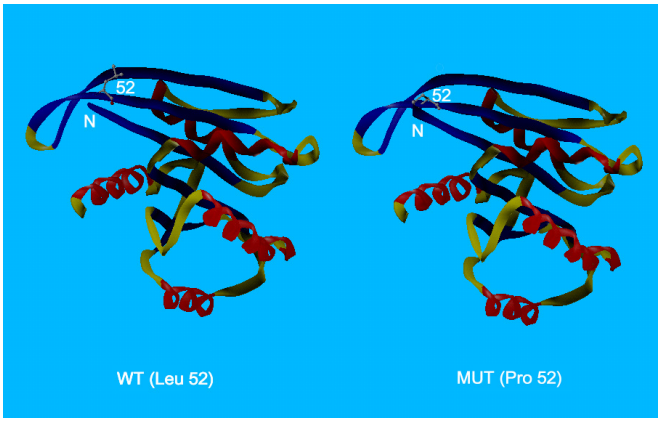

Fig. (3). Predicted structure of wt and codon 52-mutated mouse H-*ras* proteins.

DISCUSSION

6-CH_3BP has been shown to revert *S. typhimurium* strains TA98 (a -1 frameshift in a CGCGCGCG sequence in the *hisD* gene) and TA100 ($CTC^{Leu} \rightarrow CCC^{Pro}$ transition in *hisG46*) [5, 6, 31, 32]. Therefore, 6-CH_3BP can be expected to induce frameshift mutations in CG repeat sequences, and also form T.A → C.G mutations. We also observed a codon 52 (T.A → C.G) mutation in two 6-CH_3BP-induced papillomas. It is worth noting that this mutation may have been induced as A.T → G.C, rather than as T.A → C.G. Our experimental protocol cannot distinguish between these mutations. On the other hand, the codon 13 G.C → T.A mutation found in one of the seven 6-CH_3BP-induced papillomas indicates that such mutations are also formed. However, these experiments do not report the complete mutational specificity of 6-CH_3BP.

In 6-CH_3BP-treated mouse skin, two depurinating adducts, BP-6-CH_2-N7G and 6-CH_2BP-(1&3)-N7G have been identified [21]. Therefore, the H-*ras* codon 13 ($GGC^{Gly} \rightarrow GTC^{Val}$) mutation may be correlated with these Gua-depurinating adducts. At this time, the codon 52 ($CTA^{Leu} \rightarrow CCA^{Pro}$) mutation cannot be readily correlated with a particular adduct. The depurinating BP-6-CH_2-N7A adduct and the stable BP-6-CH_2-N3dT adduct are found among the *in vitro* DNA adducts formed by 6-CH_3BP [15, 17,19-21]. If these adducts are also formed in mouse skin, they could be the source of the codon 52 mutations.

The codon 52 mutation was earlier found at elevated frequencies during hyperplasia induced by dibenzo[*a,l*]pyrene (DB[*a,l*]P) in SENCAR mouse skin (~5 days after exposure) [33]. However, this mutation was not found in any DB[*a,l*]P-induced papilloma [23, 34]. Since more than one 6-CH_3BP-induced papilloma contained the same clonally-amplified codon 52 mutation, we propose that this mutation is oncogenic, and it initiated and helped establish these two tumors. This idea is consistent with previous finding that *ras* codon 52 is located in a region with transforming activity [27], and that this mutation was not predicted to induce a major structural change in the H-*ras* protein, therefore, it should retain

biological activity, and may be similar to other oncogenic *ras* proteins.

As expected, 6-CH_3BP produced a small number of skin tumors (nine papillomas in fifty-nine mice) in SENCAR mice. We analyzed seven of these nine papillomas, of which three (42.8%) contained clonally-expanded H-*ras* mutations. These numbers are significantly lower than those found with strong PAH carcinogens such as BP, 7,12-dimethylbenz[*a*] anthracene and dibenzo[*a,l*]pyrene, which show much higher incidence (up to 100%) of clonally-expanded H-*ras* mutations in papillomas [23, 34]. The small yield of tumors and the absence of the near-quantitative incidence of H-*ras* mutations in 6-CH_3BP-induced papillomas do raise the possibility that these mutations could be a consequence of the promoting treatments by TPA, rather than initiated by this PAH. Indeed, repetitive TPA treatments to SENCAR mouse skin can generate papillomas in ~20% of the animals, and some of these papillomas show H-*ras* codon 61 mutations (CAA → CGA and CAA → CTA) [35, 36]. Since 6-CH_3BP-induced papillomas did not contain any codon 61 mutations, it is more likely that the H-*ras* codon 13 and 52 mutations found in papillomas of this study were induced by this PAH.

Since 6-CH_3BP can be formed by BP bioalkylation, it has been proposed to contribute to BP tumorigenesis [12,13]. A comparison of H-*ras* mutations in BP- and 6-CH_3BP-induced papillomas provides a test of this idea at the level of initiation. Our previous studies showed that in SENCAR mouse skin, 73% BP-induced papillomas carried H-*ras* mutations, of which 50% were at codon 13 ($GGC^{Gly} \rightarrow GTC^{Val}$) and 23% were at codon 61 ($CAA^{Gln} \rightarrow CTA^{Leu}$) [23, 34]. Since no BP-induced papillomas showed the clonal codon 52 ($CTA^{Leu} \rightarrow CCA^{Pro}$) mutation, the present results are not consistent with the idea that 6-CH_3BP formation by BP bioalkylation plays a major role in initiating BP-induced tumors.

In conclusion, results indicate that 6-CH_3BP can induce oncogenic H-*ras* codon 13 and 52 mutations to initiate mouse skin papillomas. Although the limited current knowledge of 6-CH_3BP-DNA adducts in mouse skin did not permit strong stochastic relationships between DNA adducts and H-*ras* mutations, previous studies indicate that 6-CH_3BP can induce mutations of similar specificity. To our knowledge, this is the first time that a *ras* codon 52 mutation has been described as a possible oncogenic mutation. These results provide the scientific basis for studying the occurrence of this mutation in human cancer.

ABBREVIATIONS

BP = Benzo[*a*]pyrene

6-CH_3BP = 6-Methylbenzo[*a*]pyrene

PAH = Polycyclic aromatic hydrocarbons

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