Carcinogens Preferentially Bind at Methylated CpG in the p53 Mutational Hot Spots

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Abstract

The major mutational hot spots in human cancers occur at CpG sequences in the p53 gene. It is generally presumed that the majority of mutations at these sites result from the endogenous deamination of methylated cytosine. Using a UvrABC incision method, we have found that cytosine methylation greatly enhances guanine alkylation at all CpG sites in the p53 gene by a variety of carcinogens, including benzo(a)pyrene diol epoxide, benzo(g)chrysene diol epoxide, aflatoxin B1, 8,9-epoxide, and N-acetoxy-2-acetylaminofluorene. These findings suggest that mutational hot spots at methylated CpG sequences in the p53 gene may be a consequence of preferential carcinogen binding at these sites.

Introduction

Mutations in proto-oncogenes and tumor suppressor genes that are either hereditary or nonhereditary in origin are commonly observed in human cancers. Although proto-oncogenes can be activated by a variety of genetic alterations, including point mutations, the position and type of mutations that activate the oncogenic function of a proto-oncogene during carcinogenesis are generally dictated by their conveyance of selective advantages and, thus, tend to be confined to a few sequence positions with limited variations (1). A much broader spectrum and variety of signature mutations has been observed in tumor suppressor genes (1-3), which is not surprising, considering that there are more ways to abolish the function of a tumor suppressor gene product than there are ways to gain an oncogenic function from a proto-oncogene product. The frequency of mutation at each site within a gene depends upon a number of factors, including the type and frequency of DNA damage formation at that site, the rate and efficiency of repair of each type of damage, and the efficiency and fidelity of translesion synthesis by DNA polymerases; each of these factors may be affected by sequence context, and selective pressure for phenotype may result in disproportionate representation of certain mutations.

It has been found that over 50% of human cancers have a mutation in the p53 tumor suppressor gene (2). Although these mutations are distributed along the coding region of this gene at more than 200 positions, most are located in the sequences which code for amino acids in the DNA binding domain of the p53 protein (2, 3). Intriguingly, more than 30% of these p53 mutations occur at CpG sites in codons 157, 175, 245, 248, 273, and 282 (Fig. 1A), with up to 55% of the mutations in human colon cancers occurring at such sites (Fig. 1B; Refs. 2 and 3). Because all of the cytosine residues of CpG sites in the coding region of the p53 gene are known to be methylated in a variety of tissues (4), it has been hypothesized and is generally presumed that most of these mutations arise by endogenous cytosine deamination at the methylated CpG sites (5, 6). Consistent with this hypothesis is the finding that a majority of the mutations observed in human cancers are C → T transitions (2, 3, 5–7). Recently, using the UvrABC nuclease incision method in conjunction with ligation-mediated PCR techniques, we have found that, in lung cancer mutational hot spots at codons 157, 248, and 273 of the p53 gene, are the preferential binding sites for the activated cigarette smoke component BPDE (8). Our findings raise the possibility that targeted carcinogen binding rather than selective advantage is the main determinant for mutational hot spots in the p53 gene in human cancers. Our further studies have demonstrated that cytosine methylation at the CpG site determines the pattern of preferential BPDE binding at this site (9) and that BPDE adducts formed at mutational hot spots in the p53 gene are poorly repaired (10).

A significant portion of germ-line mutations in human hereditary disorders and somatic mutations in human cancers, including most of the mutational hot spots in the p53 gene, occur at CpG dinucleotide sequences (2, 3, 5–7). In light of these findings, we have determined the effect of cytosine methylation on the alkylation of guanine by various environmental and model carcinogens (11). We have used these same UvrABC nuclease reaction conditions to determine the effect of cytosine methylation at the CpG site on the guanine alkylation by the bulky chemical carcinogens BPDE, AFB1, NAAAF, and BgCDE. These compounds were chosen not only because of their carcinogenic potency (12, 13) but also because they interact with different moieties within the guanine base structure (12–15) and, therefore, allow us to probe the nature of the effects of methylation on guanine alkylation.

Materials and Methods

DNA Fragment Isolation and 32P Labeling. The p53 gene fragments containing exons 5, 7, and 8 were isolated from p53-containing plasmids (pAT153PSp, obtained from L. Crawford and S. P. Tuck, Imperial Cancer Research Fund Laboratories, London, UK) grown in Escherichia coli cells. To label DNA fragments containing exon 8 sequence, the plasmid DNAs were linearized by AvaiI digestion and then 32P-labeled at the 5' ends. The labeled DNA fragments were then digested with a second restriction enzyme, SphI, to produce single end-labeled fragments. To label DNA fragments containing exon 7 sequence, the plasmid DNAs were linearized by AvuiI and then 32P-labeled at the 5' ends. The labeled DNA fragments were then digested with a second restriction enzyme, Apai, to produce single end-labeled fragments.

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The abbreviations used are: BPDE, benzo(a)pyrene diol epoxide; NAAAF, N-acetoxy-2-acetylaminofluorene; AFB1, aflatoxin B1, 8,9-epoxide; BgCDE, benzo(g)chrysene diol epoxide; SAM, S-adenosylmethionine.
5-C Cytosine Methylation at CpG Sites. The $^{32}$P single end-labeled DNA fragments were subjected to Sssl methylase treatment with SAM to methylate all cytosines at CpG sites according to vendor's instructions.

Carcinogen Modification of Methylated and Unmethylated DNA. DNA with and without methylation treatment was then modified with BPDE ($10^{-4}$ mg/ml), BgCDE ($5 \times 10^{-3}$ mg/ml), AFB1 ($10^{-4}$ mg/ml), and NAAAF ($2.5 \times 10^{-3}$ mg/ml). The unreacted carcinogens were removed by diethyl ether extractions and by ethanol precipitation of DNA.

UvrABC Incision Assay. The adduct distributions at various sequence positions were mapped by the UvrABC nuclease incision method. In brief, UvrABC nucleuses were added to carcinogen-modified DNA fragments at a molar ratio of 6 in reaction buffer containing 100 mM KCl, 1 mM ATP, 10 mM MgCl$_2$, 10 mM Tris (pH 7.5), and 1 mM EDTA. The reactions were carried out at 37°C for 60 min and were stopped by phenol and ether extractions followed by ethanol precipitation. The resultant DNAs were denatured by dissolving in 85% formamide and heating at 90°C for 4 min and were separated by electrophoresis in 8% denaturing polyacrylamide gels.

Results and Discussion

DNA fragments containing $p53$ exon sequences were isolated from plasmids, single 5' end-labeled with $^{32}$P, and then subjected to Sssl methylase treatment with SAM to methylate all cytosines at CpG sites (9). The DNA fragments with and without methylation treatment were then modified with BPDE, NAAAF, AFB1, and BgCDE at different concentrations, and the adduct distributions at various sequence positions were mapped by the UvrABC nuclease incision method (11). The extent of cytosine methylation was determined by Maxam-Gilbert pyrimidine reaction. Because 5-C-methylated cytosines are not modified by hydrazine, both the 5' and 3' phosphodiester bonds of each methylated cytosine are refractory to piperidine hydrolysis; therefore, no cytosine ladders should be observed at methylated cytosines (16). Fig. 2A (Lane 3) shows that, under our methylation conditions, all of the CpG sites in the DNA fragment containing exon 8 sequence of the $p53$ gene are methylated. As can be seen in Fig. 2A, the guanine residues at all methylated CpG sites (codons 273, 282, 283, and 290) show great enhancement of alkylation by BPDE (Lane 8 versus Lane 7), which binds guanine at the exocyclic amine; by AFB1 (Lane 12 versus Lane 11), which binds at the N7 position; and by NAAAF (Lane 14 versus Lane 13), which binds at the C8 position (12–15). Not all CpG dinucleotides show the same enhancement for guanine alkylation; in fact, 2–5-fold variation was observed (Fig. 3). It appears that the surrounding sequences of the CpG dinucleotide may play some...
Fig. 2. The effect of cytosine methylation at the CpG dinucleotide on the binding of bulky chemical carcinogens in the human p53 gene. The p53 gene fragments containing exon 8 (A) and exon 7 (B) isolated from p53-containing plasmids were 5'-single ⁴²P end-labeled, as described in “Materials and Methods.” To methylate the cytosines at the CpG sites, DNA fragments were treated with SssI and SAM according to the vendor’s specifications (9). DNA with and without methylation treatment was then modified with BPDE (10⁻⁴ mg/ml), BgCDE (5 x 10⁻⁵ mg/ml), AFB1 (10⁻⁴ mg/ml), and NAFF (2.5 x 10⁻³ mg/ml) according to methods described previously (27-29). These modified DNA fragments were then reacted with UvrABC nuclease, and the resultant DNAs were separated by electrophoresis in an 8% denatured polyacrylamide gel, as described previously (11, 27, 28). The gel was dried and exposed to a phosphor screen. Because UvrABC nuclease incises 7 nucleotides 5' and 4 nucleotides 3' to a modified base, its incision bands are 7 bases shorter than the corresponding Maxam and Gilbert purine bands. The band intensity represents the extent of chemical modification at a particular sequence site. The sequences of the bands of interest are indicated. *C, methylated cytosine; Control, DNA without chemical carcinogen modification.
PREFERENTIAL CARCINOGEN BINDING AT CpG SEQUENCES

Fig. 3. Quantitation of the effect of cytosine methylation at CpG sites on the binding of polycyclic aromatic hydrocarbons in exon 8 (A) and exon 7 (B) of the human p53 gene. The intensity of carcinogen induced UvrABC incision bands was quantified by a PhosphorImager (Molecular Dynamics) and normalized by the amount of DNA applied in the gel. Y axis, relative intensity at different sequences, which are indicated on the X axis. Top and bottom, methylated and unmethylated DNA, respectively, modified with BPDE (a), AFB1 (b), BgCDE (c), and NAAAF (d).
role in this enhanced guanine alkylation. BgCDE, which binds at the exocyclic amine of both guanine and adenine (12–13), shows preferential binding only at the guanine residue at methylated CpG sites (Fig. 2A, Lane 10 versus Lane 9).

This cytosine methylation-enhanced carcinogen binding at the CpG sites was also observed in DNA fragments containing exon 5 (data not shown) and exon 7 (Fig. 2B) sequence. Results from Fig. 2B show that BPDE, BgCDE, AFB1, and NAAAF bind 2–5-fold higher at methylated as compared to unmethylated CpG sites at codons 245 and 248 of the p53 gene (Lane 8 versus Lane 7; Lane 10 versus Lane 9; Lane 12 versus Lane 11; and Lane 14 versus Lane 13). It is worth noting that, previously, using UvrABC incision in combination with ligation-mediated PCR, we were unable to detect BPDE-guanine adduct formation at the CpG site in codon 245, although the cytosine at this site is methylated (8). Results in Fig. 2B, in contrast, clearly demonstrate that cytosine methylation greatly enhances BPDE-guanine binding at the CpG site in this codon (Lane 8 versus Lane 7). This discrepancy is likely due to that the unknown structure at the surrounding sequences of codon 245 of the p53 gene allows UvrABC to incise BPDE adduct formed at this codon but does not allow efficient ligation with universal linker to occur, therefore resulting in poor amplification of the UvrABC incision-generated DNA fragment by the ligation-mediated PCR method. Cytosine methylation at CpG sites also has an effect on the alkylation of guanines distant from CpG sequences. Most notably, it enhances guanine alkylation at codons 272 and 276 by NAAAF, but it reduces guanine alkylation by AFB1 at codons 276 and 285 and by BPDE at codons 277 and 279.

Results presented in Figs. 2 and 3 suggest that cytosine methylation at CpG sites may dramatically change the stereo-structure and/or chemical environment of the guanines at such dinucleotide sequences; these changes may promote or enhance guanine interactions with bulky chemicals at CpG sites and either increase or decrease alkylation of guanines at sequences distant from CpG sites. It has been suggested that cytosine methylation may increase the nucleophilicity of the exocyclic amine of its paired guanine and enhance its binding with electrophilic compounds (17); however, this interpretation does not provide a satisfactory explanation for the enhanced alkylation at the N7 and C8 positions by AFB1 and NAAAF. Methylation of cytosine stabilizes (dC •¿ dG) polymer helix structure, and such methylated (dC •¿ dG) polymers have a high affinity for noncovalent binding of the nonelectrophilic compound benzo(α)pyrene tetraol (18). However, it is not known whether a single cytosine-methylated CpG dinucleotide has an increased affinity toward various electrophiles and whether enhanced noncovalent binding will lead to alkylation. Further elucidation of the cytosine methylation-induced structural changes at native sequences is needed to understand their effects on guanine alkylation.

It has been hypothesized that endogenous deamination of methylated cytosine at the CpG site may be the cause of the frequent mutations observed in human cancers (5, 6), particularly in the case of colon cancers, in which 55% of the mutations in the p53 gene occur at CpG sites (Table 1 and Fig. 1). The majority of the mutations in the p53 gene of colon cancers are C → T transitions at CpG sites, which are generally considered to be hallmarks of mutations induced by deamination of methylated cytosine (Table 1; Ref. 19). However, four important findings challenge the validity and the generality of this hypothesis. First, a significant proportion of mutations occurring at CpG sites of the p53 gene in lung, liver, head and neck, and breast involve G → T transversions (Table 1; Refs. 2 and 3); these mutations most likely are caused by mechanisms other than deamination of methylated cytosines. Second, it has been found that sequence context controls the conformation of the BPDE-DNA adduct (20, 21) and that different conformers of BPDE adducts at the same sequence may induce different types of mutation (22, 23); for example, mild heat treatment of BPDE-adducted DNA enhances G → A transitions (C → T in the complementary strand; Ref. 24), whereas polyethylene glycol treatment enhances G → T transversions (25). It is conceivable that different kinds of adducts in different sequence contexts may induce different kinds of mutations. Therefore, there is no priori reason to conclude that C → T (G → A in the complementary strand) transition at the CpG dinucleotide must be the result of cytosine deamination. Third, we have shown that cytosine methylation at CpG dinucleotides greatly enhances guanine alkylation by bulky chemical carcinogens at these sequences. Fourth, we have found that repair of bulky chemical induced DNA damage at these CpG sites in the nontranscribed strand of the p53 gene is significantly slower than repair at other sites (10). Therefore, on the basis of these findings, we propose that this higher affinity for a wide variety of DNA-damaging agents at methylated CpG sites may be the major reason that most mutations in human cancer occur at sequences containing CpG dinucleotides. It should be noted that this hypothesis does not preclude the possibility that adducts formed at the CpG sites may have higher mutability than those formed at other sequences. The unique structure at a methylated CpG site may allow a variety of chemical modifications, with differential effects on the fidelity of translation DNA synthesis. Thus, the type of mutations that occur at CpG sites may be dependent on the nature of the damaging agent and may vary for different tissues and organs. DNA-damaging agents that cause lung cancer may induce predominantly G → T transversions, but those that cause colon cancer may induce mostly G → A transitions. Therefore, a signature of G → A transition mutations or G → T transversion mutations may not be a reliable indicator of whether the mutations are due to spontaneous deamination of methylated cytosines or due to guanine modifications.

Polycyclic aromatic hydrocarbons and AFB1 contaminants are widely found in food, air, and water (12–15). Our finding that mutational hot spots in the p53 gene in human cancers have a 2–5-fold enhanced affinity toward such carcinogens strongly suggests that DNA damage induced by these agents, in addition to endogenous deamination of methylated cytosines, may be responsible for most of the mutations leading to carcinogenesis. Deamination of methylated cytosines at CpG sites has been implicated as the primary mechanism of gene dysfunction in colon cancer (6, 26). If spontaneous deamination of methylated cytosine is a major cause leading to mutations in human cancers, these mutations will be difficult to prevent. Our findings challenge these assumptions and provide further evidence that reductions in carcinogen exposure may reduce cancer risk.

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Table 1 Types of base substitution mutations at the CpG sequences of the p53 gene in different human cancers*  
<table>
<thead>
<tr>
<th>Cancer</th>
<th>C → T or G → A</th>
<th>G → T</th>
<th>G → C</th>
<th>Total mutations at CpG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>63 (35%)</td>
<td>83 (46%)</td>
<td>33 (18%)</td>
<td>35%</td>
</tr>
<tr>
<td>Liver</td>
<td>23 (48%)</td>
<td>18 (38%)</td>
<td>7 (15%)</td>
<td>18%</td>
</tr>
<tr>
<td>Head/neck</td>
<td>61 (63%)</td>
<td>23 (24%)</td>
<td>11 (12%)</td>
<td>30%</td>
</tr>
<tr>
<td>Breast</td>
<td>111 (80%)</td>
<td>17 (12%)</td>
<td>11 (8%)</td>
<td>30%</td>
</tr>
<tr>
<td>Esophagus</td>
<td>54 (64%)</td>
<td>9 (14%)</td>
<td>1 (2%)</td>
<td>40%</td>
</tr>
<tr>
<td>Colon</td>
<td>285 (94%)</td>
<td>15 (5%)</td>
<td>4 (1%)</td>
<td>55%</td>
</tr>
</tbody>
</table>

* These data were obtained from Ref. 3.

References

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