Site-Specific Mutagenesis in Human Cells by Bulky Exocyclic Amino-Substituted Guanine and Adenine Derivatives

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Purpose: 7-Bromomethylbenz[a]anthracene is a well-known mutagen and carcinogen. The aim of this study is to determine the mutagenic potency of its two major DNA adducts [N²-(benz[a]anthracen-7-ylmethyl)-2'-deoxyguanosine (b[a]a²G) and N⁶-(benz[a]anthracen-7-ylmethyl)-2'-deoxyadenosine (b[a]a⁶A)] and the simpler benzylated analogs [N²-benzyl-2'-deoxyguanosine (bn²G) and N⁶-benzyl-2'-deoxyadenosine (bn⁶A)] in Ad293 human cells and to compare to their mutagenicity in human cells and *E. coli*.

Materials and Methods: The shuttle vector pGP50 is capable of replicating in *E. coli* and human cells. Modified nucleotides were positioned in the plasmid pGP50 in a manner similar to pGP10 as described (8). Adenovirus transformed human embryonic kidney cells (line 293) were transfected with a shuttle vector containing an adduct. Two days later, the plasmids were recovered and treated with *DpnI* to remove unreplicated DNA. DH10B *E.*

coli were transformed with the plasmids. Bacteria were cultured with the media containing X-gal, IPTG and ampicillin. Bacteria transformed by the plasmid with the adduct-induced mutation in the initiation codon of *lacZ'* form white colonies whereas bacteria transformed by the plasmid without mutation form blue colonies.

Results: In the human cell site-specific mutagenesis system, bn²G exhibited weak mutagenicity and bn⁶A was not mutagenic, although b[a]a²G or b[a]a⁶A produced 8% and 7% mutant colonies, respectively. At the site of the adduct, b[a]a²G induced the G→T transversion mutation while b[a]a⁶A produced the A→G transition mutation.

Conclusion: These data indicate that bulkier b[a]a²G and b[a]a⁶A exhibit significantly greater mutagenicity in human cells than in *E. coli*. (*Cancer Research and Treatment* 2004;36:151-156)

Key Words: Mutagenesis, Mutagen

INTRODUCTION

Mutation has been recognized to initiate carcinogenic processes. The initial event in this multistage process is the covalent modification of cellular DNA by various reactive chemical carcinogens (1).

It has been difficult to characterize the mechanism of an individual carcinogen-DNA adduct altering and damaging the structure or coding properties of DNA since chemical carcinogens can react with multiple sites on DNA bases as well as with several DNA bases simultaneously. Thus, the chemical synthesis of DNA sequences and plasmids containing a single carcinogen-modified base at a predetermined sequence site has been used as a probe for the analysis of the structural and biological effects of defined carcinogen-modified bases (2,3).

The environmental pollutant 7-bromomethylbenz[a]anthracene (7-BrMeBA), an electrophile acting directly, is a well-known mutagen and carcinogen (4 ~ 7). N²-(benz[a]anthracen-7-ylmethyl)-2'-deoxyguanosine (b[a]a²G) and N⁶-(benz[a]anthracen-7-ylmethyl)-2'-deoxyadenosine (b[a]a⁶A) are the major deoxyribonucleosides in DNA modified by 7-BrMeBA. The site-specific mutagenicity of the major DNA adducts produced by 7-BrMeBA, i.e., b[a]a²G and b[a]a⁶A, when the modified base was incorporated into either a double-stranded or gapped plasmid vector was previously examined in *E. coli* (8). This study revealed that these bulky aralkylated adducts did not exhibit significant mutagenicity, which was unexpected.

Several studies showed the difference in the mutagenic potency of exocyclic DNA adducts between *E. coli* and mammalian cells, which indicates that the mutation frequency was influenced by host cells (9 ~ 11). These results led to the comparison of the mutagenic potency induced by b[a]a²G and b[a]a⁶A in *E. coli* and human cells and to examine the mutagenicity of simpler benzylated derivatives of guanine (N²-benzyl-2'-deoxyguanosine, bn²G) and adenine (N⁶-benzyl-2'-deoxyadenosine, bn⁶A) (12) to assess the effect of the size of the exocyclic amino group substituent on mutagenic potency.

Here, this study reports the site-specific mutagenesis induced by four aralkylated nucleosides b[a]a²G, b[a]a⁶A, bn²G, and

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Received February 12, 2004, Accepted March 24, 2004

bn⁶A (Fig. 1) in human embryonic kidney cells (line 293). In addition, this study compares the mutagenic potency of these four aralkylated nucleosides in human cells and *E. coli*.

MATERIALS AND METHODS

The restriction enzymes were obtained from New England Biolabs, Beverly, MA and were dissolved in the buffer supplied by the manufacturer unless specified. T4 polynucleotide kinase, T4 DNA ligase, uracil DNA glycosylase, X-gal, and IPTG were purchased from USP Corp., Cleveland, OH. All other enzymes and reagents used in this study were described previously (13). Adenovirus-transformed human embryonic kidney cell (Ad293) (14) was obtained from Otsuka Pharmaceutical, Rockville, MD. 2'-Deoxyguanosine and 2'-deoxyadenosine were purchased from Sigma Chemical Co., St. Louis, MO. Most other reagents and solvents were purchased from Aldrich Chemical Co., Milwaukee, WI. 7-Bromomethylbenz[a]anthracene was prepared as described previously (15). N²-benzyl-2'-deoxyguanosine, N⁶-benzyl-2'-Deoxyadenosine, N²-(benz[a]anthracen-7-ylmethyl)-2'-deoxyguanosine, N⁶-(benz[a]anthracen-7-ylmethyl)-2'-deoxyadenosine and these bulky aralkylated adducts containing 16-base oligodeoxyribonucleotides were prepared as described (12). Synthetic DNA sequences containing N²-aralk¹-ated guanine and N⁶-aralkylated adenine nucleosides, 5'-d^{*}(A C A G C C A T A* T G* G C C C)-3' and a complementary 16-mer 5'-C U U G G G G C C A U A U G G C-3' were prepared by a DNA synthesizer (Applied Biosystems, Inc., Model 380B) and purified by polyacrylamide gel electrophoresis. All oligonucleotides were characterized by enzymatic digestion and followed by chromatographic analysis as described previously (12).

1) Preparation of adduct containing plasmids

A pair of oligonucleotides were incorporated into the human mutagenesis shuttle vectors pGP50 using the procedure described previously (Fig. 2) (13,16~18). In these constructions, vector DNA was digested with *Bsp*MI restriction enzyme to remove the insert DNA sequence shown in the box in Fig. 2. The major fragment produced by *Bsp*MI digestion was isolated and recircularized by ligating a pair of oligonucleotides as illustrated in Fig. 2 into the plasmid where the insert had been. Following the recircularization, covalently closed plasmids were isolated.

2) Mutagenesis experiments in *E. coli*

The mutagenic potency induced by four bulky aralkylated adducts in *E. coli* was determined as described previously (8,16).

3) Mutagenesis experiments in human cells

The human embryonic kidney cells (line 293, ATCC CRL 1573) were transfected with the shuttle vector containing control oligonucleotide or the adduct containing oligonucleotides (Fig. 3). Cells were incubated for 48 hours to allow the replication of the shuttle vector. Subsequently, plasmid DNA was recovered and treated with *Dpn*I to remove unreplicated vectors. The recovered plasmids were used to transform the alpha complementing *E. coli* strain DH10B (19). *E. coli* thus transformed were cultured with the media containing X-gal and IPTG and blue or white colonies were scored. The procedures

for the growth and transfection of human cells, the recovery and *Dpn*I treatment of the vectors, and transformation and growth of *E. coli* have been described previously (19).

4) Sequencing of mutant plasmids

To characterize the adduct containing the *lacZ'* inactivating mutation and control plasmids, white colonies were isolated and subcultured. Plasmid DNA was isolated and the region surrounding the *lacZ'* initiation codon was sequenced using a dye terminator cycle sequencing kit (Perkin Elmer, Foster City, CA) and a DNA sequencer (Applied Biosystems 373A, Foster City, CA)

RESULTS

1) Mutagenicity in *E. coli*

The significant mutation frequency was not detected by aralkylated adducts [bn²G, bn⁶A, b[a]a²G, and b[a]a⁶A] in the *E. coli* site-specific mutagenesis system as reported previously (8) (Fig. 4).

2) Mutagenicity in human cells

The site-specific mutation induction of the major DNA adducts produced by the aralkylating agent 7-bromomethyl benz[a]anthracene [b[a]a²G and b[a]a⁶A] and the simpler benzylated analogs (bn²G and bn⁶A) in the adenovirus- transformed

Fig. 1. Chemical structure of four bulky aralkylated nucleosides.

Fig. 2. The shuttle vector pGP50 is capable of replicating in *E. coli* and human cells. SV40 ori makes pGP50 capable of replicating in human cells. T-antigen is required to utilize the SV40 ori. Modified nucleotides were positioned in this plasmid in a manner similar to pGP10 as described (8).

human embryonic kidney cells Ad293 were examined.

pGP50 is a shuttle vector capable of replicating in both *E. coli* and human cells. Modified nucleotides were positioned in the plasmid pGP50 in a manner similar to pGP10 as described (8,13). Adenovirus transformed human embryonic kidney cells (line 293) were transfected with the adduct containing the shuttle vector. Two days later, plasmids were recovered and treated with *DpnI* to remove unreplicated DNA. The plasmids were used to transform DH10B *E. coli*. DH10B *E. coli* bacteria were cultured with the media containing X-gal, IPTG and ampicillin. The site-specific mutagenesis induced by the bulky adducts was evaluated by the sector colony assay system as previously described (13). Bacteria with the adduct-induced mutation in the initiation codon of *lacZ'* grow as white colonies. Bacteria without mutation grow as blue colonies.

In the human cell site-specific mutagenesis system, DH10B cells transformed with the plasmid derived from the b[a]a²G containing gapped vector that has been replicated in 293 cells produced 8% mutant colonies. Similarly, DH10B cells transformed with the plasmid derived from b[a]a⁶A produced 7% mutant colonies. In contrast, bn²G exhibited weak mutagenicity and bn⁶A was not mutagenic (Fig. 5).

3) Mutations in human cells

Thirty white colonies were grown following the tranfor-

mation with control plasmids or the plasmids harboring the adduct. Plasmid DNA was isolated and sequenced. In cells transformed with bn²G, two transversions, G-to-T, and one transition, G-to-A, were detected at the site of the adduct. The base change at the site of bn⁶A was not detected. In cells transformed with b[a]a²G seventeen transversions, G-to-T were detected at the site of the adduct. In cells transformed with b[a]a⁶A, on the other hand, 17 A-to-G transitions were detected at the site of the adduct (Table 1).

DISCUSSION

Recently, the site-specific mutagenesis in *Escherichia coli* by four bulky exocyclic amino-substituted guanines and adenines [bn²G, bn⁶A, b[a]a²G and b[a]a⁶A] incorporated site-specifically to the ATG initiation codon of the *lacZ'* gene in both double-stranded and gapped plasmids were reported (8). In this *E. coli* site-specific mutagenesis system, these alkylated adducts did not exhibit the significant mutagenicity. The SOS induction also had no measurable effect on the mutagenicity of these adducts. The result that could not induce mutation in *E. coli* was totally unexpected since the environmental pollutant 7-bromomethylbenz[a]anthracene (7-BrMeBA) is a well-known mutagen and carcinogen. These data strongly suggest that the site-specific mutagenesis by these four bulky adducts should be

examined in human cells. This study thus evaluated the site-specific mutagenicity of the major DNA adducts produced by the alkylating agent 7-BrMeBA, [b[a]a²G and b[a]a⁶A] in human cells, when the modified base was incorporated into plasmid vectors and subsequently compared the mutagenic potency in *E. coli* and human cells. In addition, the effect of the size of the exocyclic amino group substituent on the mutagenic potency was examined by comparing the mutagenicity of b[a]a²G and b[a]a⁶A with the analogous but simpler benzylated derivatives of guanine and adenine [*N*²-benzyl-2'-deoxyguanosine (bn²G) and *N*⁶-benzyl-2'-deoxyadenosine (bn⁶A)].

The mutagenicity of b[a]a²G and b[a]a⁶A in human cells was substantially higher than in *E. coli* (Fig. 4, 5). Several studies have reported the marked differences in the mutagenic potency of the exocyclic DNA adducts between *E. coli* and mammalian cells (9~11). 1,*N*⁶-ethenodeoxyadenosine was highly mutagenic in mammalian cells but marginally mutagenic in *E. coli* (9). 1,*N*²-ethenodeoxyguanosine, on the other hand, was highly mutagenic in *E. coli* but only weakly mutagenic in mammalian cells. The converse relationship was observed for 3,*N*⁴-ethenodeoxycytidine (10). The present study results together with these reports suggest that the mutation frequency is markedly influenced by host cells.

In addition, the bulkier b[a]a²G and b[a]a⁶A were signifi-

cantly more mutagenic than simpler adducts (bn²G and bn⁶A) in human cells. This indicates that the size of the exocyclic amino group substituent has profound effect on the mutagenic potency of bulky adducts in the site-specific mutagenesis. These data broaden our understanding of the mechanism of substituted structure influencing the mutagenicity of the adduct with bulky exocyclic amino-substitution in human cells.

The mutaton induced by 7-BrMeBA was examined in the *supF* gene of the plasmid pS189 previously (20). Ad293 cells were transfected with the 7-BrMeBA-modified pS189 DNA and the plasmid was extracted. The extracted DNA was used to transform the *E. coli* MBM 7070 cells. In this evaluation system, the mutagenic potency of 7-BrMeBA was substantial and

Fig. 3. Adenovirus transformed human embryonic kidney cells (line 293) were transfected with the shuttle vector containing an adduct. The recovered shuttle vectors were used to transform *E. coli* where the adduct-induced mutation was scored by the loss of *lacZ'* activity producing white colonies.

Fig. 4. Colony phenotypes in *E. coli*, resulting from alkylated nucleosides in dU-containing plasmids or gapped plasmids. The value represents the mean±SE of three experiments.

Fig. 5. Colony phenotypes resulting from alkylated nucleosides in human cells. The difference in mutagenicities was analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range t-test. A significant difference in mutagenicities between alkylated nucleosides and control is indicated by **P* < 0.05. The value represents the mean±SE of three experiments.

Table 1. The base change induced by bulky N²-aralkylated guanines or N⁶-aralkylated adenines in human cells

<p style="text-align: center;">bn²G</p> <p>5'-AACAGCCATATGGCCC-3'</p> <p style="text-align: center;">T T A</p> <p style="text-align: center;">b[a]a²G</p> <p>5'-AACAGCCATATGGCCC-3'</p> <p style="text-align: center;">: T : (17/30)</p>	<p style="text-align: center;">bn⁶A</p> <p>5'-AACAGCCATATGGCCC-3'</p> <p style="text-align: center;">b[a]a⁶A</p> <p>5'-AACAGCCATATGGCCC-3'</p> <p style="text-align: center;">: G : (17/30)</p>
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the predominant base substitution was the G→T transversion. The present study results are in agreement with these reported results (Table 1).

The sequence context effect (21 ~ 24) and efficient repair (16 ~ 18) have been assumed as the causality of the failure of these bulky aralkylated adducts [bn²G, bn⁶A, b[a]a²G and b[a]a⁶A] to induce significant mutation in *E. coli*. However, the adducts have the strikingly different mutagenic potency in *E. coli* and human cells. The significant mutagenicity of these adducts may be intrinsic to their structure in human cells.

CONCLUSIONS

In this human cell site-specific mutagenesis system, bn⁶A was not mutagenic and bn²G was only weakly mutagenic. In contrast, b[a]a²G and b[a]a⁶A were highly mutagenic. In addition, bulkier b[a]a²G or b[a]a⁶A exhibit significantly greater mutagenicity in human cells than in *E. coli*, which further emphasize the importance of studying the site-specific mutagenesis by carcinogen-modified DNA bases in human cells.

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