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AFB₁-induced mutagenesis of the *gpt* gene in AS52 cells

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Abstract

Aflatoxin B₁ (AFB₁) is a potent mutagen and an important risk factor for hepatocellular carcinoma (HCC) in humans. Transgenic mouse strains and cells in culture have been used to detect different types of mutations caused by AFB₁ and investigate the molecular determinants of their location and frequency. The AFB₁ mutational spectrum in the *gpt* gene was markedly different in AS52 cells compared to the liver in *gpt* delta B6C3F1 transgenic mice. The results demonstrate the importance of metabolism, chromosomal location, transcription and selection conditions on mutational spectra.

Keywords

mutation spectrum; xgprt; 6-thioguanine

1. Introduction

Aflatoxin B₁ (AFB₁) is a highly mutagenic and carcinogenic metabolite of the mold *Aspergillus flavus*, which is a common contaminant of food crops. AFB₁ is carcinogenic in many animal species and has been identified as a major risk factor for the development of hepatocellular carcinoma in exposed populations in China and Africa [El-Serag and Rudolph 2007].

Mutational spectra induced by AFB₁ have been examined in bacteria, shuttle vectors and endogenous genes in mammalian cells [Cariello *et al.* 1994; Foster *et al.* 1983; Levy *et al.* 1992]. Several groups have recently determined mutational spectra induced by AFB₁ in bacterial reporter genes in transgenic B6C3F1 mouse liver [Chen *et al.* 2010; Woo *et al.* 2011].

The development of transgenic mice with integrated bacterial reporter genes has made it possible to characterize and quantify spontaneous mutations in organs and tissues as well as mutagenic changes that occur as a consequence of chemical exposures. The reporter transgenes are not expressed and therefore may not be subject to selection bias *in vivo* enabling accurate comparisons of the ability of environmental chemicals to produce genetic

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changes in the reporter gene [Heddle *et al.* 2003]. The fact that these genes are not transcribed and are generally present in multiple tandem arrays within chromosomes, however, raises uncertainty about their representation of the types of mutations produced in actively transcribed genes that are crucial for normal growth and development.

The pSV2*gpt* gene complex originally constructed by Mulligan and Berg can be expressed in *E. coli*, rodent and human cells [Mulligan and Berg 1981; Tindall *et al.* 1984]. AS52 cells are hypoxanthine-guanine phosphoribosyltransferase-deficient CHO AA8 cells transformed with pSV2*gpt* and carrying a single chromosomally integrated copy of the *gpt* gene that expresses xanthine-guanine phosphoribosyltransferase [Tindall *et al.* 1984; Tindall *et al.* 1986]. Spontaneous and induced *gpt* mutants can be directly selected on the basis of resistance to 6-thioguanine (6-TG) toxicity..

The bacterial *gpt* gene is also the reporter gene in the *gpt* delta B6C3F1 mouse [Nohmi *et al.* 1996]. In this transgenic system, the *E. coli gpt* gene flanked by loxP sites was incorporated into a λ phage integrated into chromosomal DNA. The haploid mouse genome contains approximately 80 copies of the recombinant λ EG10 phage located in tandem on chromosome 17 [Masumura *et al.* 1999b]. The xanthine-guanine phosphoribosyltransferase product of the *gpt* gene is not expressed in mouse tissues, but rescue of the integrated phage and infection of Cre⁺ *E. coli* results in expression of the protein. As in AS52 cells, selection of 6-TG^r *E. coli* permits isolation and analysis of mutated *gpt* gene sequences.

There have been several direct comparisons of *in vivo* mutagenesis at endogenous and transgenic loci within transgenic animals [Skopek *et al.* 1996; Walker *et al.* 1996; Walker *et al.* 1999]. The availability of AS52 cells and the *gpt* delta B6C3F1 mouse provided the opportunity to compare the mutational spectra of AFB₁ in a single gene in different genomic contexts. In addition to chromosomal context of the gene sequence, selection conditions for 6-TG^r mutants could also bias the mutational spectra. In the *gpt* delta mouse transgenic system, selection of 6-TG^r mutants occurs after *gpt* expression in *E. coli*, while *gpt* expression in AS52 cells is the basis for mutant selection. Thus, the purpose of this study was to characterize the mutational profile induced by AFB₁ in the *gpt* gene in AS52 cells for comparison with that induced *in vivo* in the *gpt* delta B6C3F1 mouse in our previous work [Woo *et al.* 2011].

2. Materials and methods

2.1 Cell culture and chemicals

AS52 cells were cultured in Ham's F-12 medium, containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine (BioWhittaker, Walkersville, MD) in a humidified atmosphere of 5% CO₂ at 37°C. Pre-existing 6-thioguanine resistant (6-TG^r) mutants were eliminated by growth for 7 days in the above medium plus 10 μ g/ml mycophenolic acid, 250 μ g/ml xanthine, 22 μ g/ml adenine, 11 μ g/ml thymidine and 1.2 μ g/ml aminopterin [Tindall and Stankowski, Jr. 1989]. Thereafter, cells were grown for 3 days in Ham's F-12 medium containing with penicillin, streptomycin and L-glutamine plus 11.5 μ g/ml xanthine, 3 μ g/ml adenine and 1.2 μ g/ml thymidine. Cells were used immediately or stored frozen at -80°C.

Aflatoxin B₁ (AFB₁), dimethylsulfoxide (DMSO), nicotinamide adenine diphosphate (NADP) and isocitric acid were obtained from Sigma-Aldrich (St. Louis, MO). Human liver S9 fraction was obtained from BD Biosciences (San Jose, CA).

2.2 AFB₁ metabolic activation system

Cells were treated with AFB₁ in the presence of a metabolic activation system prepared as follows: Ham's F12 medium containing penicillin, streptomycin and L-glutamine was combined with pooled human liver S9 fraction (0.44 mg protein/ml), NADP (1.6 mg/ml) and DL-isocitric acid (2.9 mg/ml). The solution was adjusted to neutral pH with 1 N NaOH.

AS52 cells, cleansed of *gpt* mutants, were seeded at a density of 5×10^5 cells/well in 6-well plates, grown for 24 h and washed with serum-free medium followed by addition of serum-free medium containing the metabolic activation system. AFB₁ dissolved in DMSO was then added to cell cultures to achieve final concentrations of 1 to 32 μ M; DMSO concentrations did not exceed 1%. Controls received DMSO only. Cultures were incubated at 37°C for 5 h, washed with serum-free medium and cultured in complete growth medium for 24 h. All treatments were carried out in triplicate.

2.3 Cytotoxicity and mutation assays

After 24 h cells were trypsinized and cytotoxicity assessed by trypan blue exclusion. Thereafter, treated cells were maintained in growth medium for at least 7 days to allow full expression of the *gpt* mutant phenotype. To select 6-TG^r cells, 5×10^5 cells were suspended in 100 ml of medium containing 10 μ M 6-thioguanine (6-TG) and seeded at a density of 5×10^4 cells/100 mm dish (10 dishes/group). Simultaneously, cells were seeded at 2.5×10^3 cells/100 mm dish (5 dishes/group) under non-selective conditions to estimate plating efficiency (PE). After a 2 wk incubation, colonies were stained with 0.5% crystal violet in 50% methanol and counted. PE and mutant frequency (MF) were calculated as follows:

$$PE = \text{Number of colonies} / \text{Number of seeded cells} \quad MF = \text{Number of 6-TG}^r \text{ colonies} / \text{Number of seeded cells} \times PE$$

2.4 PCR amplification and sequencing analysis of the *gpt* gene

Sequencing of the *gpt* gene for mutation spectrum analysis was performed on 6-TG^r clones from DMSO control or AFB₁-treated AS52 cells. Genomic DNA was isolated using the GenElute™ mammalian genomic DNA miniprep kit (Sigma-Aldrich). Nested-PCR amplification of individual *gpt* mutants was performed using Phusion® High-Fidelity DNA Taq polymerase (New England BioLabs). PCR conditions were those used in previous studies [Ferguson *et al.* 1998; Tindall and Stankowski, Jr. 1989] with modifications. Briefly, the primary PCR reaction used forward 5'-AAGCTTGGACACAAGACAG-3' and reverse 5'-CCAGAATACTTACTGGAAAC-3' primers. Agarose gel electrophoresis of PCR products identified the 0.7 kb *gpt* gene and a 1.1 kb non-coding rearrangement of the pSV2gpt vector [Tindall and Stankowski, Jr. 1989]. The 0.7 kb product was purified for DNA sequence analysis using the QIAquick gel extraction kit (Qiagen) and concentrated with a Microcon® YM-30 column (Millipore). DNA sequencing was carried out by Dana-Farber/Harvard Cancer Center DNA Resource (Boston, MA) with primers 5'-ATAACAGGCTGGGACACTT-3' and 5'-AGTGCCAGGCGTTGAAAAGA-3'.

3. Results

AFB₁ treatment in the presence of human liver S9 fraction was both cytotoxic and mutagenic to AS52 cells (Fig. 1). AFB₁ induced a dose-dependent decrease in cell survival which was reduced to 35% at the highest concentration (32 μ M). Induction of 6-TG^r mutants by AFB₁ was also dose-dependent at concentrations up to 8 μ M (Fig. 1). The mutant fraction (MF) in DMSO-treated cultures was 2.8×10^{-5} , consistent with previous reports for the spontaneous MF in AS52 cells [Tindall and Stankowski, Jr. 1989]. At 8 μ M AFB₁ the 6-TG^r MF was 44×10^{-5} , a 16-fold increase over DMSO-treated cells; higher concentrations

did not increase the 6-TG^r MF further. A total of 290 independent 6-TG^r colonies from control (DMSO) and AFB₁-treated cultures were isolated for characterization of the mutations in the *gpt* gene.

Sequence analysis of 6-TG^r clones from AS52 cells treated with either DMSO or AFB₁ revealed that approximately 60% did not have mutations in the coding region of the *gpt* gene. In this group of mutants the genetic change(s) conferring the 6-TG^r phenotype likely occurred outside the structural sequence of the gene or in other genes that result in resistance to 6-TG.

Deletions represented the majority of sequence changes identified in 6-TG^r AS52 cells following treatment with DMSO. A 3-basepair deletion at positions 379-371 in the *gpt* gene was the predominant mutation (50%) found in this control group. This observation is consistent with the high prevalence of small deletions between bases 366 and 387 of the *gpt* gene observed in several cell lines carrying an integrated *gpt* gene [Ashman and Davidson 1987; Tindall and Stankowski, Jr. 1989]. Single base-pair substitutions (35%), and one base-pair deletions (13%) were the next most prevalent spontaneous mutants in the *gpt* coding region. The most common single base-pair substitutions were G:C to T:A (27%) transversions and G:C to A:T (9%) transitions, along with G:C to C:G (9%) and A:T to C:G (5%) transversions (Table I). In AFB₁-treated AS52 cells for which changes in the structural *gpt* gene sequence were found, single base substitutions represented 78% of the mutations while 18% contained deletions (8% of 1 bp and 10% >1 bp) (Table I). Single base pair insertions were also present (3%). The predominance of G to T transition mutants (65% of total) is consistent with the mutational properties of the AFB₁-DNA adducts that have been identified in other assay systems [Bailey *et al.* 1996; Levy *et al.* 1992; Smela *et al.* 2001].

The distributions of mutations within the *gpt* gene in AS52 cells treated with either DMSO (control) or AFB₁ are shown in Figure 2. At nucleotides 30 (5'-GAC-3'), 107 (5'-AGC-3'), 115 (5'-GGT-3') and 418 (5'-GAT-3') G:C to T:A or C:G to A:T transversions represented 5% of mutations within the *gpt* coding region. These nucleotides could either be preferentially damaged by AFB₁, sites that are difficult to repair, or result in amino acid substitutions that are most effective in inactivating XGPRT. Some of the mutations found in AFB₁-treated cells are likely of spontaneous origin considering that they include 4 instances of the 3-base pair deletion occurring at position 369 to 371. The most prevalent genetic change among 6-TG^r clones in DMSO-treated cells.

Differences between the AS52 cell and transgenic mouse *gpt* mutational spectra obtained in a previous study are shown in Table II. Nucleotide changes at 12 positions in the *gpt* gene differed between the two spectra. Statistical comparison using Monte Carlo type analysis [Adams and Skopek 1987] showed that the mutational spectrum induced by AFB₁ in the *gpt* gene of AS52 cells was significantly different ($p < 0.0001$) from that induced in liver of *gpt* delta B6C3F1 mice [Woo *et al.* 2011].

4. Discussion

AS52 cells contain a single copy of the *gpt* gene that is expressed *via* the SV40 early promoter [Tindall *et al.* 1986]. In B6C3F1 *gpt* delta mice the transgene is not expressed. Approximately 80 copies of the λ EG10 phage containing the *E. coli gpt* reporter gene are integrated in tandem at a single locus on chromosome 17 [Masumura *et al.* 1999a]. Chromosomal integration of the *gpt* gene in both experimental models provides an opportunity to compare the mutagenic specificity of AFB₁ in the same gene located within different genomic contexts. Findings reported here reveal significant differences between

locations of mutations in AS52 cells with those we observed in a previous study employing the *gpt* transgene in the liver of AFB₁-treated *gpt* delta B6C3F1 mice [6].

In AS52 cells, base pair substitutions were the most frequent AFB₁-induced mutations in the *gpt* gene. These occurred exclusively at G:C base pairs with G:C to T:A transversions accounting for the major type. This transversion is the predominant mutation induced by AFB₁-N⁷-guanine or AFB₁-FAPY adducts in several *in vitro* experimental systems [Bailey *et al.* 1996; Foster *et al.* 1983; Smela *et al.* 2001]. Deletions were the second most frequent AFB₁-induced mutation. Single base deletions, mostly at G:C sites and several larger deletions in the GC-rich 200–210 bp segment of the *gpt* gene resulted from AFB₁ treatment. The 3-base deletion at bps 369–371 found after AFB₁ treatment was also identified in DMSO-treated cells, most likely a result of spontaneous 6-TG^r mutants within the treated population.

The different molecular and chromosomal contexts of the *gpt* gene in AS52 cells and mouse liver showed evidence of preferential damage by AFB₁ in one strand. Alkylating compounds display both regioselectivity and sequence selectivity for reaction with nucleophilic centers in DNA. The 8,9-AFB₁ oxide reacts with the N7 position of guanine in the major groove, preferentially modifying guanines flanked by G+C-rich sequences [Benasutti *et al.* 1988; Muench *et al.* 1983]. Mutations occurring at nucleotides 106–108 (5'-AGC-3') in AS52 cells showed G:C to T:A or C:G substitutions at position 107, the second base pair (5'-AGC-3'). In the B6C3F1 mouse liver, however, C:G to A:T or G:C substitutions were found exclusively at the third base pair of this triplet, position 108 (5'-AGC-3'); likely the result of an AFB₁-modified guanine in the opposite strand.

The *gpt* selection system had a strong influence on the composition of induced and spontaneous mutational spectra. Resistance to 6-TG toxicity requires efficient inactivation of XGPRT [Thilly *et al.* 1978]. Consequently, conditions for selection of 6-TG^r mutants favor base substitutions that produce a stop codon or an amino acid substitution that prevents substrate binding or interferes with catalytic activity. Similarities would therefore be expected to exist in *gpt* mutational spectra in AS52 cells and the *gpt* delta B6C3F1 mouse. In both spectra, about 50% of mutations occurred within 3 clusters (bps 45–60; 100–140 and 400–420) that comprise 16% of the structural *gpt* sequence. Nonetheless, within these clusters the types of mutations found in the *gpt* gene appeared to be strongly influenced by the environment in which 6-TG^r cells were selected. The different base substitutions at nucleotides 107 or 108 resulted in codon changes that replaced a serine with either threonine or isoleucine in Xgp^rt expressed in 6-TG^r AS52 cells, while in transgenic *gpt* delta B6C3F1 mice, serine was replaced by either arginine or cysteine in 6-TG^r *gpt* mutants selected in *E. coli*. Furthermore, 25% of base substitutions resulted in stop codons in the *gpt* gene obtained from transgenic mice, while they represented <10% of mutations in 6-TG^r AS52 cells. These results indicate that both the genomic context and selective environment strongly influence the types of AFB₁-induced mutations observed in the *gpt* sequence.

Most spontaneous mutants in the *gpt* gene in AS52 cells have been reported to be deletions [Stankowski, Jr. *et al.* 1986]. In our experiments, approximately 50% of 6-TG^r mutants in DMSO-treated cultures contained a 3-base deletion at bp 369–371, a previously identified mutational hot-spot [Tindall and Stankowski, Jr. 1989]. The limited number of spontaneous base substitution mutants analyzed does not permit statistical comparison with other groups, but none were characteristic of those previously reported to occur at 5'-CpG-3' mutational hot-spots [Masumura *et al.* 2000].

A large fraction of 6-TG^r clones had no change in the *gpt* structural gene sequence. Changes in methylation at CpG sites in the SV40 early promoter within the integrated pSV2-*gpt*

construct could result in gene silencing leading to 6-TG resistance [Bryans *et al.* 1992]. In prior studies with other agents, this group of 6-TG mutants in AS52 cells has been attributed to the abundance of small repeated sequences flanking the *gpt* integration site that could be a source of recombination events resulting in gene silencing [Ferguson *et al.* 1998; Stankowski, Jr. *et al.* 1986; Tindall and Stankowski, Jr. 1989]. AFB₁ has been shown to be a strong inducer of mitotic recombination at the thymidine kinase locus in human lymphoblasts (TK6) [Stettler and Sengstag 2001]. It is also possible that AFB₁-induced changes in the expression of other genes required for 6-TG toxicity could be responsible resistant cells lacking *gpt* mutations. For example, expression of S-methyltransferases can decrease 6-TG toxicity by producing metabolites that are not good substrates for the salvage pathway [Wang and Weinshilboum 2006]. Once incorporated into DNA, the lethal effects of 6-TG depend on processing 6-TG DNA by the mismatch repair (MMR) system, which provokes strand breaks that can activate cell cycle checkpoint responses [Karran and Attard 2008]. Thus, if AFB₁ treatment affected MMR efficiency corresponding changes in sensitivity towards 6-TG would be expected.

A quantitative comparison of the induced MF in the *gpt* gene in AS52 cells and *gpt* delta B6C3F1 mice is not possible because we do not know the AFB₁ concentrations to which hepatocytes in the *gpt* delta B6C3F1 mouse were exposed, only the administered dose. The extent of metabolic conversion of AFB₁ to its reactive 8,9-oxide is also unknown so it is difficult to compare precise exposure conditions experienced by liver hepatocytes to those of AS52 cells. Previous studies have reported large differences in the abilities of S9 fractions from chick embryo, rat liver and human liver to bioactivate AFB₁ and increase the *gpt* MF in AS52 cells [Goeger *et al.* 1998; Goeger *et al.* 1999]. Although mutational spectra were not examined in these studies, investigations of other mutagens provide evidence that metabolic differences can produce significant variations in mutational spectra [Chen and Thilly 1996; Wei *et al.* 1999]. Measurement of the amount of AFB₁-N⁷-guanine in the DNA of both cell types would provide a metric of exposure since the extent of its formation has been directly related to AFB₁ dose in experimental animals and humans [Groopman *et al.* 2002].

MF can also be influenced by the ability of cells to repair damaged bases, which varies with chromosomal location, sequence context and the activation of stress response pathways. Enhanced rates of repair have been observed for DNA damage in the transcribed strand of expressed genes [Hanawalt 1989], while DNA lesions in nonexpressed heterochromatin have slower repair rates [Bohr *et al.* 1987; Leadon *et al.* 1983]. Prior work has shown that the rate of removal of AFB₁ adducts is slower in highly repeated DNA sequences compared to other regions of the genome [Leadon *et al.* 1983]. A large difference between induced MFs in the *lacI* transgene and *hprt* loci in B6C3F1 Big Blue mice has been attributed to different rates of DNA repair in the transcribed *Hprt* gene vs the nontranscribed *lacI* transgene [Skopek *et al.* 1996]. Furthermore, activation of checkpoint pathways can recruit error-prone translesion synthesis polymerases that enhance the potential of mutagenic lesions in DNA [Koren 2007; McCulloch and Kunkel 2008]. The induction of error prone DNA polymerases by the SOS response in *E. coli* has been found to increase the mutagenicity of the AFB₁-DNA adducts [Bailey *et al.* 1996; Smela *et al.* 2001]. Further studies are needed to clarify the role of checkpoint and stress responses in mammalian cells to AFB₁ mutagenicity.

The AFB₁-induced *gpt* mutational spectrum from AS52 cells may prove relevant in relating the results from *in vitro* assays to those obtained in transgenic animal systems and exposed humans. Comparisons of mutational spectra between different loci can reveal factors that govern the sensitivity of tissues, cells and endogenous genetic loci to mutation and the selection of genetically altered cells during the process of tumorigenesis. The frequency and

nature of mutants induced in genetic loci in different genomic contexts will lead to a better understanding of the factors that determine the sensitivity of mutational targets. Our results also point to a need for additional investigations of the mutagenic and recombinogenic activities of AFB₁ in transgenic animals to understand the full range of its genotoxic potential.

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Figure 1. Effect of AFB₁ on growth (**top**) and induction of 6-TG^r mutants (**bottom**) in AS52 cells. Cells were treated with AFB₁ for 4 h in the presence of human liver S9. Values are means $\pm$ SD; n = 3.

Figure 2.
Nucleotide sequence of the *gpt* gene indicating position and type of mutation induced by DMSO (red, above) or AFB₁ (black, below). Y = insertion; □ = deletion.

Table ITypes of mutations observed in AS52 cells treated with AFB₁ or DMSO

Mutation Types	AS52	
	DMSO	AFB ₁
Transition:		
G:C to A:T	2(4)	4(3)
A:T to G:C	0(0)	0(0)
Transversion:		
G:C to T:A	7(13)	77 (65)
G:C to C:G	9(16)	10(8)
A:T to T:A	0(0)	2(2)
A:T to C:G	1(2)	0(0)
Deletion		
1 bp	7(13)	10(8)
>1 bp	28 (50)	12(10)
Insertion	2(4)	4(3)
Total number of mutants	56(100)	119 (100)

() % of total mutants

Table II

Differences between the AFB₁-induced mutational spectra in the *gpt* gene in AS52 cells and liver of *gpt* delta B6C3F1 mice

AFB ₁ -induced changes in <i>gpt</i> sequence		
Nucleotide	AS52	<i>gpt</i> delta B6C3F1 [†]
1		C→A (1)
3		G→T (1)
7		G→T (2)
22	1 bp deletion (2)	
30	C→A (4); 1 bp deletion (3)	
49	C→A (4)	
50	G→A (1); G→A (1); G→C (2)	
101		C→A (5)
107	G→T (5); G→C (1)	
108		C→A (8); C→G (3); C→T (1)
145		G→T (4); G→A (1)
189		C→A (3)
242	G→T (2)	
369-371	3 bp deletion (4)	
376	G→T (3)	
401		G→T (3); G→C (1)
402		G→A (2); G→T (1)
406		G→A (4); G→T (3)
412	C→A (2)	
413	C→T (1)	
414	G→T (3)	

[†]Data from Woo et al. 2011 [6] included for comparison; mice treated with 6 mg/kg AFB₁; () number of mutants