Mutational Spectrum and Genotoxicity of the Major Lipid Peroxidation Product, *trans*-4-Hydroxy-2-nonenal, Induced DNA Adducts in Nucleotide Excision Repair-Proficient and -Deficient Human Cells†

Zhaohui Feng,‡ Wenwei Hu,‡ Shantu Amin,§ and Moon-shong Tang*,‡,||,⊥

Departments of Environmental Medicine, Medicine, and Pathology, New York University School of Medicine, Tuxedo, New York 10987, and Division of Carcinogenesis and Molecular Epidemiology, American Health Foundation, Valhalla, New York 10595

Received March 18, 2003; Revised Manuscript Received April 29, 2003

ABSTRACT: *trans*-4-Hydroxy-2-nonenal (4-HNE), a major product of lipid peroxidation, is able to interact with DNA to form 6-(1-hydroxyhexanyl)-8-hydroxy-1,N^2-propano-2′-deoxyguanosine (4-HNE−dG) adducts, but its genotoxicity and mutagenicity remain elusive. It has been reported that 4-HNE treatment in human cells induces a high frequency of G·C to T·A mutations at the third base of codon 249 (AGG*) of the *p53* gene, a mutational hot spot in human cancers, particularly in hepatocellular carcinoma. This G·C to T·A transversion at codon 249, however, has been thought to be caused by etheno−DNA adducts induced by the endogenous metabolite of 4-HNE, 2,3-epoxy-4-hydroxynonanal. We have recently found that 4-HNE preferentially forms 4-HNE−dG adducts at the GAGG*C/A sequence in the *p53* gene including codon 249 (GAGG*C). Our finding supports the possibility that G·C to T·A mutations at codon 249 may be induced by 4-HNE−dG adducts. To investigate this possibility, we determined the mutational spectrum induced by 4-HNE−dG adducts in the *supF* gene of shuttle vector pSP189 replicated in human cells. We have found that 4-HNE−dG adducts are mutagenic and genotoxic in human cells, and that G·C to T·A transversions are the most prevalent mutations induced by 4-HNE−dG adducts. Furthermore, 4-HNE−dG adducts induce a significantly higher level of genotoxicity and mutagenicity in nucleotide excision repair (NER)-deficient human and *Escherichia coli* cells than in NER-proficient cells, indicating that NER is a major pathway for repairing 4-HNE−dG adducts in both human and *E. coli* cells. Together, these results suggest that 4-HNE−dG adducts may contribute greatly to the G·C to T·A mutation at codon 249 of the *p53* gene, and may play an important role in carcinogenesis.

Lipid peroxidation is a common cellular process that becomes significant when cells are under oxidative stress, exposed to xenobiotics, and subjected to bacterial and viral infection (*1*−*3*). A growing body of evidence has shown that lipid peroxidation is involved in carcinogenesis (*2*−*6*). For example, rats treated with a choline-deficient diet or peroxisome proliferators, each of which induces lipid peroxidation, developed liver tumors (*7*, *8*). Moreover, it has been shown that many carcinogens and tumor promotion agents can stimulate lipid peroxidation (*9*−*12*). Lipid peroxidation can produce many species of aldehydes, including *trans*-4-hydroxy-2-nonenal (4-HNE,[1] Figure 1), acrolein, crotonaldehyde, and malondealdehyde; of that group, 4-HNE is the most abundant and cytotoxic (*1*). 4-HNE is formed by radical-initiated degradation of ω-6-polyunsaturated fatty acids such as linoleic and arachidonic acids, two relatively abundant fatty acids in human cells (*1*). Although 4-HNE can be further metabolized to 2,3-epoxy-4-hydroxynonanal (EH) in cells, which can interact with DNA to form exocyclic etheno−guanine, etheno−adenine and etheno−cytosine adducts (*13*), a significant amount of 6-(1-hydroxyhexanyl)-8-hydroxy-1,N^2-propano-2′-deoxyguanosine (4-HNE−dG, Figure 1), a bulky exocyclic DNA adduct caused by the direct interaction of 4-HNE with DNA, has been found in various normal tissues of humans and rats (*14*−*18*). It has also been found that the amount of 4-HNE−dG adduct significantly increases in the livers of rats treated with carbon tetrachloride, a condition which can induce lipid peroxidation and liver carcinogenesis in rats (*17*, *19*). These results suggest a potentially important role that 4-HNE and 4-HNE−dG adducts may play in mutagenesis and carcinogenesis.

Recently, we have discovered that 4-HNE preferentially forms 4-HNE−dG adducts at the GAGG*C/A sequence in

† Supported by Public Health Service Grants ES03124, ES08389 (M.-s.T.), and P30-CA 17613 (S.A.) and Service Core support from NIEHS Center Grants ES10344 and ES00260.

* To whom correspondence should be addressed. Phone: (845) 731-3585. Fax: (845) 351-2385. E-mail: tang@env.med.nyu.edu.

‡ Department of Environmental Medicine, New York University School of Medicine.

§ American Health Foundation.

|| Department of Medicine, New York University School of Medicine.

⊥ Department of Pathology, New York University School of Medicine.

[1] Abbreviations: 4-HNE, *trans*-4-hydroxy-2-nonenal; 4-HNE−dG, 6-(1-hydroxyhexanyl)-8-hydroxy 1,N^2-propano-2′-deoxyguanosine; NER, nucleotide excision repair; EH, 2,3-epoxy-4-hydroxynonanal; HCC, hepatocellular carcinoma; SDS, sodium dodecyl sulfate; LB, Luria broth; IPTG, isopropyl β-D-thiogalactoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; BPDE, benzo[*a*]pyrene diol epoxide; CCC, covalently closed circle; OC, open circle.

10.1021/bi034431g CCC: $25.00
Published on Web 06/06/2003

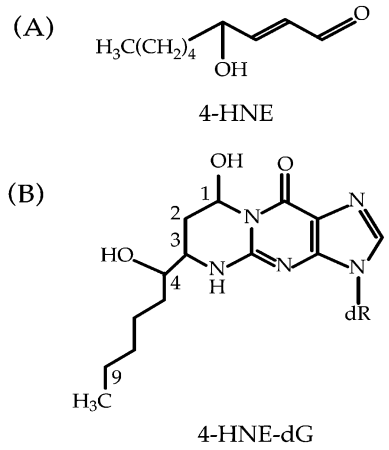

FIGURE 1: Chemical structures of *trans*-4-hydroxy-2-nonenal (4-HNE) and the 6-(1-hydroxyhexanyl)-8-hydroxy 1,N^2-propano-2′-deoxyguanosine (4-HNE−dG) adduct.

the human *p53* gene, including codon 249 (GAGG*C) (*20*). It has been reported that exposure of wild-type *p53* human lymphoblastoid cells to 4-HNE causes a high frequency of G·C to T·A transversion mutation at the third base of codon 249 (AGG*) of the human *p53* gene (*21*). The G·C to T·A transversion mutation at codon 249 of the *p53* gene is a mutational hot spot in human cancers, and is the most prevalent mutation found in hepatocellular carcinoma (HCC) in patients infected with hepatitis B virus in the regions contaminated with aflatoxin B1 (*22*−*24*). Interestingly, we (unpublished data), as well as others (*25*), have found that activated aflatoxin B1 does not bind strongly at codon 249 of the *p53* gene in cultured human cells. In the woodchuck model, it has been found that animals exposed to both hepatitis B virus and aflatoxin B1 have increased levels of lipid peroxidation in their livers, and these animals eventually develop HCC (*26*). Together, these results raise the possibility that 4-HNE plays an important role in hepatocarcinogenesis.

To date, the mutagenicity and genotoxicity of 4-HNE and 4-HNE−dG adducts remain elusive. It has been reported that 4-HNE is the most cytotoxic and least mutagenic among the aldehydes produced by lipid peroxidation (*1*, *18*, *27*, *28*). This conclusion is mainly derived from the results of treating cultured mammalian cells and bacteria with exogenous 4-HNE (*1*, *18*, *27*, *28*). Compared with other bulky DNA-damaging agents such as benzo[*a*]pyrene diol epoxide (BPDE), 4-HNE produces much lower levels of DNA damage at the doses that exhibit similar cytotoxicity in cells (*1*, *20*, *29*). 4-HNE is known to be reactive to macromolecules such as proteins with a thiol group; hence, the exogenous 4-HNE-induced cytotoxicity is likely mainly due to its interactions with macromolecules other than genomic DNA (*1*, *2*). The extreme cytotoxicity and tissue toxicity of exogenous 4-HNE have hampered the assessment of 4-HNE−DNA adduct formation and its repair in cultured cells and animal models. To avoid the strong non-4-HNE−dG-induced cytotoxic effects of 4-HNE, in this report we have modified the shuttle vector pSP189 with 4-HNE to produce 4-HNE−dG adducts and to assess their genotoxicity and mutagenicity in nucleotide excision repair (NER)-proficient and -deficient human cells.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. NER-proficient *Escherichia coli* (*E. coli*) strain MST1 (*thr*-1, *leu*-6, *proA*2, *his*-4, *argE3* or arg-49, *lac* Y1, *galK*2, *rspL*31, or *rpsL*154, *supE*44) and NER-deficient *E. coli* strains MST13 (*uvrA6*) and MST3 (*uvrB5*) were grown in Luria broth (LB) as described previously (*30*). The shuttle vector pSP189, which contains the tyrosine suppressor tRNA coding gene *supF* as a mutational target, was kindly provided by M. Seidman (National Institute on Aging, National Institutes of Health, Baltimore, MD) and purified as described previously (*31*).

Cell Culture. SV-40-transformed NER-deficient human XPA fibroblasts (XP12BE) and NER-proficient human fibroblasts (GM00637) were obtained from the NIGMS Human Genetic Cell Repository (Camden, NJ). These cells were grown in minimum essential medium supplemented with 10% fetal bovine serum in a 5% CO_2 humidified incubator.

4-HNE Modification of Plasmid DNA. 4-HNE was synthesized according to the method described previously (*32*). Plasmid pSP189 DNA was modified with 4-HNE as previously described (*20*). In brief, a stock solution was prepared by dissolving 4-HNE in methanol at a concentration of 100 mg/mL. The purified plasmid DNA was dissolved in TE buffer [10 mM Tris and 1 mM EDTA (pH 7.0)], mixed with various concentrations of a 4-HNE solution, and incubated at 37 °C for 30 h. Control plasmids were treated with methanol only. The unreacted 4-HNE was removed by repeated phenol and diethyl ether extractions, and the treated plasmids were then ethanol precipitated and dissolved in TE buffer (pH 7.0). We have previously shown that under these modification conditions the 4-HNE−dG adduct is the major DNA adduct that is formed (*20*).

UvrABC Incision Assay of 4-HNE−DNA Adducts. UvrABC nuclease, the NER protein complex of *E. coli*, was purified from *E. coli K12* strain CH296 carrying plasmid pUNC 45 (*uvrA*), pUNC21 (*uvrB*), or pDR3274 (*uvrC*). These plasmids and strain CH296 were kindly provided by A. Sancar (University of North Carolina, Chapel Hill, NC). The purification procedures were the same as previously described (*33*). Standard UvrABC incision assays (*20*, *34*) were carried out in a 25 μL solution containing 100 mM KCl, 1 mM ATP, 10 mM $MgCl_2$, 10 mM Tris (pH 7.5), and 1 mM EDTA. UvrABC nuclease was added to 4-HNE-modified plasmids at a molar ratio of 6:1 (UvrABC nuclease:plasmid DNA). The reactions were stopped after 60 min at 37 °C by adding 0.1% sodium dodecyl sulfate (SDS) and heating at 65 °C for 5 min; the plasmid DNA was then separated by electrophoresis in a 1% agarose gel in TAE buffer [40 mM Tris-acetate (pH 8.0) and 1 mM EDTA] at 1 V/cm overnight, stained with ethidium bromide (0.5 μg/mL), and visualized with UV light. The number of UvrABC nuclease incisions in a 4-HNE-modified plasmid was calculated on the basis of the Poisson distribution equation $P(0) = e^{-n}$, where $P(0)$ represents the fraction of covalently closed circle (CCC) plasmid DNA after UvrABC incision and n represents the average number of UvrABC incisions per plasmid DNA.

Transformation of E. coli Cells. The pSP189 plasmids with and without 4-HNE modifications were electroporated into NER-proficient *E. coli* cells (MST1) and NER-deficient *E. coli* MST13 (*uvrA6*) and MST3 (*uvrB5*) cells using the

E. coli Pulser (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. Transformed *E. coli* cells were plated on LB plates containing ampicillin (50 μg/mL). After overnight incubation at 37 °C, bacterial colonies were counted, and the relative transformation frequencies were calculated with the formula N/N_0, where N is the number of colonies formed per unit of 4-HNE-modified plasmids and N_0 is the number of colonies formed per unit of unmodified plasmids.

Transfection of Cultured Human Cells. The pSP189 plasmids with and without 4-HNE modifications were transfected into human NER-deficient XPA fibroblasts (XP12BE) and NER-proficient fibroblasts (GM00637) using Lipofectamine (Invitrogen, Carslbad, CA), according to the manufacturer's instructions. Briefly, 3×10^6 cells were plated into 150 mm tissue culture dishes in culture medium and incubated overnight, and the cells were transfected with a mixture of plasmids (40 μg) and Lipofectamine (Invitrogen) for 72 h. The transfected plasmids were then rescued from the human cells by the alkaline lysis method as described by Lee et al. (*35*). Cells were trypsinized, washed, resuspended in suspension buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 100 μg/mL RNase A], mixed with lysis buffer (0.2 M NaOH and 1% SDS), and incubated on ice for 15 min, followed by the addition of neutralization buffer [3 M potassium acetate (pH 5.5)]. After incubation at room temperature for 15 min, the mixture was centrifuged for 10 min at 16000*g*, and the supernatant was extracted with phenol and chloroform, and precipitated with ethanol. The DNA was resuspended and treated with the *Dpn*I restriction enzyme (New England BioLabs, Beverly, MA) to remove the unreplicated plasmids which bear the bacterial adenine methylation pattern. The replicated plasmids were then electroporated into indicator MB7070 bacteria, which carry a *lacZ* gene with an amber mutation. The transformed bacteria were plated on LB plates containing ampicillin (50 μg/mL), isopropyl β-D-thiogalactoside (IPTG) (190 μg/mL), and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (0.8 mg/mL). After overnight incubation at 37 °C, white and light blue mutant colonies were picked from the background of blue wild-type colonies and restreaked; the plasmids were then extracted and purified using the QIApre-spin plasmid kit (Qiagen, Valencia, CA). The sequences of the *supF* gene of mutant plasmids were determined with the primer 5′-GGC GAC ACG GAA ATG TTG AA-3′ and the CEQ dye terminator cycle sequencing kit (Beckman Coulter, Fullerton, CA) using the CEQ 2000XL Automatic DNA analyzer (Beckman Coulter).

RESULTS

Genotoxicity of the 4-HNE−dG Adduct in NER-Proficient and -Deficient E. coli and Human Cells. To address the question of 4-HNE−dG adduct-induced genotoxicity, we have chosen the shuttle vector−host cell transfection system in this study to avoid the strong non-4-HNE−dG-induced cytotoxic effects of 4-HNE. Previously, we have demonstrated that the 4-HNE−dG adduct is the major DNA adduct produced when DNA is reacted with 4-HNE under neutral pH conditions (*20*), and that UvrABC nuclease, the NER protein complex isolated from *E. coli*, is able to cut this type of adduct quantitatively and specifically (*20*). Utilizing UvrABC nuclease, we quantified the number of 4-HNE−dG adducts in the 4-HNE-modified shuttle vector pSP189. The results shown in Figure 2 indicate that the amount of UvrABC nuclease cutting, which represents the number of 4-HNE−dG adducts in pSP189 plasmids, is proportional to the concentrations of 4-HNE used for plasmid modification. These modified plasmids were then transformed into both NER-proficient and -deficient *E. coli* and human cells. As shown in Figures 3 and 4, the relative transforming frequency of the 4-HNE-modified pSP189 plasmids in both NER-proficient *E. coli* and human cells is much higher than in their NER-deficient counterparts. The presence of one 4-HNE−dG adduct in the pSP189 plasmids reduces the relative transforming frequency to 40 ± 8 and 43 ± 7% of that of the unmodified control pSP189 plasmids in NER-deficient *E. coli* (*uvrA* and *uvrB*) cells and human XPA cells, respectively, while reducing the relative transforming frequency to 83 ± 7 and 90 ± 6% of that of the unmodified pSP189 plasmids in NER-proficient *E. coli* and human cells. These results suggest that (1) the 4-HNE−dG adduct is as genotoxic as other bulky DNA adducts, such as the BPDE−dG adduct, which has been reported to efficiently block DNA replication (*29*, *36*), and (2) NER is a major pathway for the repair of 4-HNE−dG adducts in both *E. coli* and human cells.

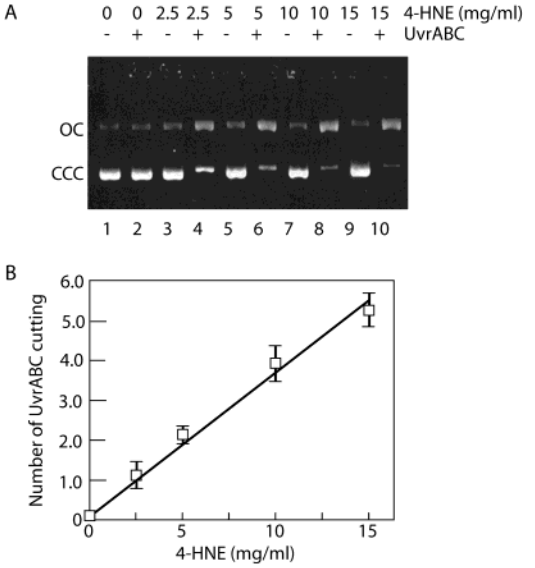

FIGURE 2: UvrABC incision of 4-HNE-modified supercoiled pSP189 plasmids. Supercoiled pSP189 plasmid DNA was modified with various concentrations of 4-HNE and then reacted with (lanes 2, 4, 6, 8, and 10) or without (lanes 1, 3, 5, 7, and 9) UvrABC nuclease. The resultant plasmid DNA was separated by electrophoresis in a 1% agarose gel. (A) A typical gel: OC, open circle; and CCC, covalently closed circle. (B) Quantitation. The number of UvrABC nuclease incisions in a 4-HNE-modified plasmid was calculated on the basis of the Poisson distribution equation $P(0) = e^{-n}$, where $P(0)$ represents the fraction of CCC plasmid DNA after UvrABC incision and n represents the average number of UvrABC incisions per plasmid DNA. Data are from three independent experiments.

Mutagenicity of the 4-HNE−dG Adducts in Human Cells. It has been reported that 4-HNE is a poor mutagen in both bacterial cells and *lacI* transgenic animal models when treated with exogenous 4-HNE (*1*, *18*, *27*, *28*, *37*). Since exogenous 4-HNE is a potent cytotoxin and tissue toxin, the low mutagenicity of 4-HNE in these systems may be due to the extreme toxic effects of 4-HNE, and the inefficient DNA

Table 1: 4-HNE-Induced Mutation Frequencies of *supF* in pSP189 Plasmids Replicated in NER-Deficient XPA Fibroblasts and NER-Proficient Human Fibroblasts[a]

		NER-deficient (XPA)		NER-proficient (GM00637)	
4-HNE (mg/mL)	no. of adducts per plasmid	no. of mutants/no. of total colonies	mutation frequency ($\times 10^4$)	no. of mutants/no. of total colonies	mutation frequency ($\times 10^4$)
0	0	16/133526	1.2 ± 0.3	19/49850	3.8 ± 0.4
5	2.1 ± 0.2	64/32735	19.6 ± 1.0	52/46428	11.2 ± 0.8
10	3.8 ± 0.4	113/21638	52.5 ± 2.2	92/33192	27.6 ± 1.3
15	5.3 ± 0.4	206/26185	78.7 ± 3.2	155/33610	46.2 ± 2.5

[a] The statistical difference between two cell lines is $p < 0.005$ (χ^2 test).

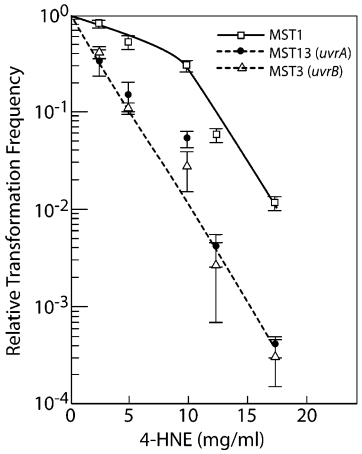

FIGURE 3: Relative transformation frequency of 4-HNE-modified pSP189 plasmids in NER-proficient (MST1) and NER-deficient [MST13 (*uvrA6*) and MST3 (*uvrB5*)] *E. coli* cells. The 4-HNE-modified pSP189 plasmids were used to transform NER-proficient (MST1) and NER-deficient (MST13 and MST3) *E. coli* cells. Transformed *E. coli* cells were selected by ampicillin resistance on LB plates. The relative transformation frequency was calculated on the basis of the formula N/N_0, where N is the number of colonies formed per unit of 4-HNE-modified plasmids and N_0 is the number of colonies formed per unit of control plasmids. Data are from three independent experiments.

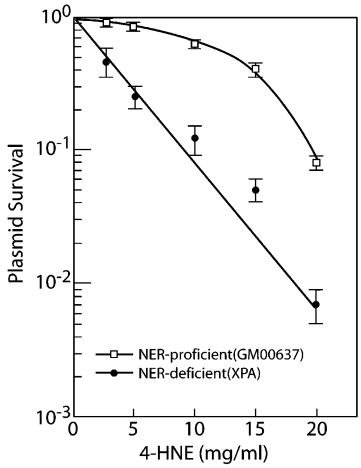

FIGURE 4: Survival of 4-HNE-modified pSP189 plasmids in NER-proficient (GM00637) and NER-deficient (XPA) human fibroblasts. The 4-HNE-modified pSP189 plasmids were transfected into NER-proficient (GM00637) and NER-deficient (XPA) human fibroblasts. After replication in cells for 72 h, the progeny plasmids were harvested and used to transform indicator MBM7070 bacteria. The level of plasmid survival was calculated on the basis of the formula N/N_0, where N is the number of colonies formed per unit of 4-HNE-modified plasmids and N_0 is the number of colonies formed per unit of unmodified control plasmids. Data are from three independent experiments.

adduction by exogenous 4-HNE (*28*). To overcome the extreme cytotoxicity of 4-HNE, we have transfected pSP189 shuttle vectors containing different amounts of 4-HNE−dG adducts into human cells. This allows us to directly determine the mutagenicity of the 4-HNE−dG adducts without the influence of cytotoxicity. 4-HNE-modified pSP189 shuttle vectors were transfected into NER-deficient and -proficient human fibroblast cells. The progeny plasmids were rescued and transformed into indicator bacteria MBM7070 to identify plasmids carrying mutations in the *supF* gene. The mutation frequency of *supF* was defined as the ratio of the number of white or light blue mutant colonies to the total number of bacterial colonies obtained on indicator plates containing IPTG and X-Gal. Results in Figure 5 and Table 1 show that the mutation frequencies of *supF* increase linearly with the numbers of 4-HNE−dG adducts in the plasmids in both NER-deficient and -proficient human fibroblast cells. Plasmids with 4-HNE−dG adducts produce significantly higher *supF* gene mutation frequencies in NER-deficient XPA cells than in NER-proficient human fibroblast cells ($p < 0.005$). The mutagenicity (mutation frequency per adduct in plasmids) of 4-HNE in both NER-proficient and -deficient human cells is similar to that of BPDE−dG adducts (*36*, *38*). These results suggest that 4-HNE−dG adducts are highly mutagenic, and also suggest that NER is a major pathway for the repair of 4-HNE−dG adducts in human cells.

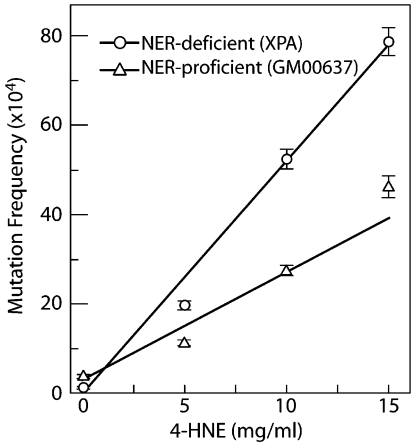

FIGURE 5: Mutation frequency of the *supF* gene in 4-HNE-modified pSP189 plasmids replicated in NER-proficient (GM00637) and NER-deficient (XPA) human fibroblasts.

Mutational Spectrum of supF in NER-Deficient and -Proficient Human Cells. It has been shown that in cultured human lymphoblasts, 4-HNE treatment induces a high frequency of G·C to T·A transversions at the third base of codon 249 of the *p53* gene (*21*), a mutation frequently observed in HCC from individuals infected with hepatitis B virus in regions contaminated with aflatoxin B1 (*22*−*24*).

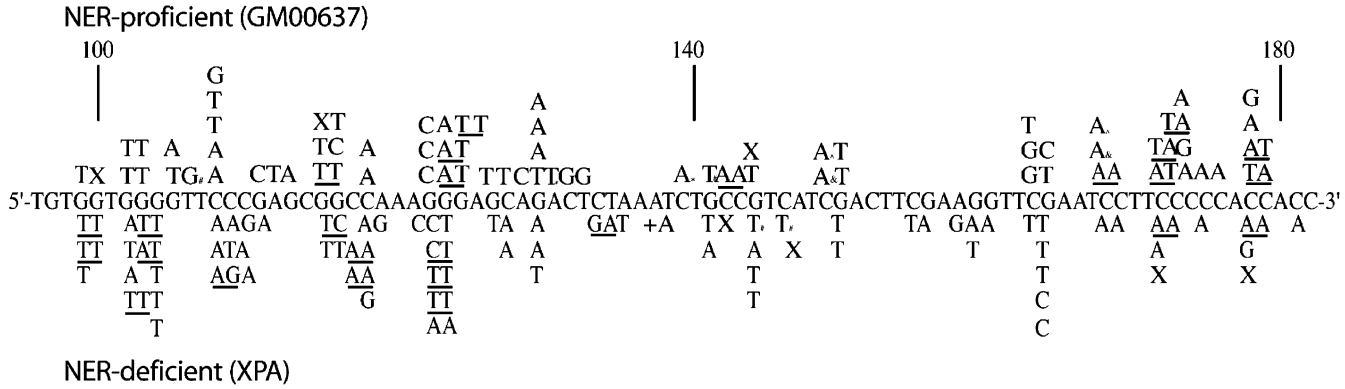

FIGURE 6: Mutational spectrum of the *supF* gene in 4-HNE-modified pSP189 plasmids replicated in NER-proficient (GM00637) (top panel) and NER-deficient (XPA) (bottom panel) human fibroblasts. Tandem mutations (mutations occur at two adjacent bases) are underlined. X represents a single-base deletion. +A denotes single-base insertions. The symbols #, &, ∧, and * denote multiple mutations (two mutations occur in the same plasmid).

This transversion mutation at codon 249 of the *p53* gene has been thought to be induced by exocyclic etheno−dG adducts produced by the interaction of DNA with EH, the endogenous metabolite of 4-HNE in cells, rather than by the 4-HNE−dG adducts produced by the direct interaction of DNA with 4-HNE (*21*). However, it has been demonstrated that exocyclic etheno−dG adducts induce G·C to A·T transitions rather than G·C to T·A transversions in *E. coli* (*2*, *39*). In light of the importance of lipid peroxidation in hepatocellular carcinogenesis, we have determined the mutational spectrum induced by 4-HNE−dG adducts in NER-deficient and -proficient human cells by sequencing the *supF* gene in 79 and 66 independent mutant pSP189 plasmids recovered after the 4-HNE-modified pSP189 plasmids have been passed through NER-deficient XPA and NER-proficient human fibroblast cells, respectively. The type of mutations and the mutational spectrum induced by 4-HNE−dG adducts in the *supF* gene in NER-proficient and -deficient cells are presented in Table 2 and Figure 6. There are five features worth noting. (1) 4-HNE−dG adducts induce the same kinds of mutations in both NER-proficient and -deficient cells, and there is no statistical difference in mutation types between NER-deficient and -proficient human fibroblast cells. These results indicate that the mutations are likely to be the result of replication errors rather than nucleotide excision repair errors. (2) The majority of observed mutations are base substitutions (94% in NER-deficient cells and 96% in NER-proficient cells) with more than 70% being single-base substitutions, and the majority (90%) of these base substitutions occur at guanine or cytosine sites. (3) Like other bulky DNA adducts such as the BPDE−dG adduct, the majority (50%) of mutations induced by the 4-HNE−dG adduct in both NER-deficient and -proficient cells are G·C to T·A transversions. (4) 4-HNE−dG adducts induce a high frequency of tandem mutations (mutations occur at two adjacent bases) in both NER-proficient (17%) and NER-deficient cells (19%), and most of the tandem base substitutions occur at GG or CC sites. (5) Mutations are clustered at sites with contiguous G and/or C bases.

Table 2: Types of Mutations in the *supF* Gene in 4-HNE-Treated pSP189 Plasmids Replicated in NER-Deficient XPA Fibroblasts and NER-Proficient Human Fibroblasts

	NER-deficient (XPA)		NER-proficient (GM00637)	
	control	treated[a]	control	treated[a]
single base substitution				
G·C to T·A	5 (38%)	29 (50%)	5 (36%)	23 (48%)
G·C to A·T	5 (38%)	20 (35%)	5 (36%)	10 (21%)
G·C to C·G	2 (15%)	6 (10%)	3 (21%)	11 (23%)
A·T to T·A	1 (8%)	0 (0%)	0 (0%)	2 (4%)
A·T to C·G	0 (0%)	2 (3%)	1 (7%)	2 (4%)
A·T to G·C	0 (0%)	1 (2%)	0 (0%)	0 (0%)
total	13	58	14	48
base substitution[b]				
G·C to T·A	5 (38%)	54 (59%)	5 (36%)	40 (51%)
G·C to A·T	5 (38%)	22 (24%)	5 (36%)	17 (22%)
G·C to C·G	2 (15%)	10 (11%)	3 (21%)	12 (15%)
A·T to T·A	1 (8%)	1 (1%)	0 (0%)	6 (8%)
A·T to C·G	0 (0%)	2 (2%)	1 (7%)	3 (4%)
A·T to G·C	0 (0%)	1 (1%)	0 (0%)	0 (0%)
total	13	90	14	78
single-base substitution	13 (88%)	58 (73%)	14 (88%)	48 (73%)
single-base deletion	1 (6%)	4 (5%)	2 (12%)	3 (4%)
single-base insertion	1 (6%)	1 (1%)	0 (0%)	0 (0%)
tandem mutation[c]	0 (0%)	15 (19%)	0 (0%)	11 (17%)
multiple mutation[d]	0 (0%)	1 (1%)	0 (0%)	4 (6%)
total	15	79	16	66

[a] Plasmids were treated with 15 mg/mL 4-HNE. [b] Including single-base substitution, tandem mutation, and multiple mutation. [c] Mutations occur at two adjacent bases. [d] Two mutations occur in the same plasmid.

DISCUSSION

There is a growing body of evidence that suggests that the endogenous DNA damage induced by lipid peroxidation may play an important role in carcinogenesis (*2*−*6*). Although 4-HNE is one of the most abundant aldehyde products of lipid peroxidation, the role of 4-HNE and 4-HNE−dG adducts in mutagenesis and carcinogenesis remains unclear. It has been shown that 4-HNE is able to induce hypoxanthine-guanine phosphoribosyltransferase (HPRT)-deficient mutations and sister-chromatid exchanges in Chinese hamster cells (*40*, *41*); however, it has been reported that 4-HNE lacks mutagenic activity in bacterial mutagenicity tests using *Salmonella* tester strains and shows no genotoxicity in *lacI* transgenic animal models (*27*, *37*). The reasons why the mutagenic and carcinogenic activities of 4-HNE and, in particular, 4-HNE−DNA adducts have remained obscure are probably as follows. (1) Treatment with exogenous 4-HNE results in high cytotoxicity and tissue toxicity, which in turn limits the use of either cells or animal models for mutagenicity tests. (2) Difficulties in producing and detecting 4-HNE−DNA adducts exist. (3) The formation of 4-HNE−

DNA adducts exhibits sequence context preferences. We have recently found that 4-HNE preferentially forms 4-HNE−dG adducts at special sequences (GAGG*C/A) in the human *p53* gene, and the binding at this special sequence could be more than 10-fold stronger than that at other sequences (*20*). This special sequence for 4-HNE−DNA adduct formation is not present in the *Salmonella* tester strains which carry targeted sequences such as CCC, CCCCCC, CGCGCGCG, or ATT for detecting his^- to his^+ backward mutations (*27*, *42*, *43*). In addition to the high cytotoxicity of 4-HNE, this factor may also contribute to the lack of mutagenic activity of 4-HNE in *Salmonella* tester strains.

The exogenous 4-HNE can be further metabolized to EH in mammalian cells, and the latter can interact with DNA to form exocyclic etheno−guanine, etheno−adenine, and etheno−cytosine adducts, like many other aldehydes (*13*, *18*, *27*). EH and its DNA adducts have been shown to induce mutations in both bacterial and mammalian cells, and it is likely that the formation of etheno−DNA adducts via the EH metabolite may be one mechanism of 4-HNE-induced mutagenicity and genotoxicity (*13*, *18*, *27*). In this study, however, we addressed directly the mutagenicity and genotoxicity of 4-HNE−dG adducts. Previously, we have shown that the 4-HNE−dG adduct is the major DNA adduct formed under the conditions we used for 4-HNE modification of DNA (*20*). Consistent with this conclusion are our current results which show that 90% of 4-HNE−DNA adduct-induced mutations occur at G·C sites. Only 5 and 12% of mutations occur at A·T sites in *supF* in NER-deficient and -proficient cells, respectively, and these A·T sites are immediately adjacent to G·C sites (Table 2 and Figure 6). These mutations could result from the minor 4-HNE−DNA adducts formed at A·T sites, or caused by nontargeted mutations induced by 4-HNE−dG adducts at adjacent guanines. Compared with the mutational spectrum induced by 4-HNE−dG adducts in NER-deficient XPA cells, the mutational spectrum in NER-proficient human cells is similar, but the mutations occur at a much lower frequency ($p < 0.005$). These results indicate not only that NER is error free for the repair of 4-HNE−dG adducts but also that the fidelity of 4-HNE−dG replication in both NER-proficient and -deficient cells is similar.

It is worth noting that 17−19% of the 4-HNE−DNA adduct-induced mutations were tandem mutations, which mainly occurred at GG or CC sites. Among these tandem mutations, G·C to T·A transversion mutations are the major mutations with 24 of 30 (80%) in NER-deficient cells and 13 of 22 (60%) in NER-proficient cells. Using the same shuttle vector system for determining the mutational spectrum, it has been found that acrolein and crotonaldehyde also induce a similar high level of tandem mutations at GG or CC sites (*44*, *45*). Since acrolein is able to induce intrastrand cross-links between adjacent guanine bases, it has been suggested that this type of intrastrand cross-links contributes to the formation of tandem mutations (*44*). It is possible that 4-HNE is able to induce intrastrand cross-links and subsequently induces tandem mutations.

Except the frequent CC to TT mutations in skin cancers (*46*) and very few cases in non-small lung carcinomas (*47*) and esophageal tumors (*48*), tandem mutations are rarely observed in the *p53* gene of human cancers, including liver cancer (*46*). However, frequent tandem mutations, in particular G·C to T·A transversions, have been observed in the livers and adipose tissue of aging transgenic mice harboring the *lacI* gene (*49*). These tandem mutations may be caused by the metabolites of lipid peroxidation since aldehydes induce a high frequency of tandem mutations. It has been found that only the codon 249-mutated *p53* gene product has a strong dominant-negative effect in liver cells; that is, liver cells with a mutation at codon 249 have stronger *p53*-minus phenotypes, such as a reduced level of transcription transactivation, increased resistance to apoptosis, and higher propensity to be carcinogenic, than liver cells with a mutation at other codons (*50*−*52*). The reasons why codon 249 of the *p53* gene is a mutational hot spot in human HCC while no tandem mutations are seen in the *p53* gene are perhaps due to the fact not only that is codon 249 the preferential site for 4-HNE binding but also that liver cells with a mutation at this site have a growth advantage.

In summary, our results clearly demonstrate that 4-HNE−DNA adducts are mutagenic and genotoxic in human cells and induce a high frequency of G·C to T·A transversion mutations, and NER is the major pathway for repairing 4-HNE−DNA adducts. Since we have found that 4-HNE preferentially binds at codon 249 of the human *p53* gene, it is likely that 4-HNE−dG DNA adducts contribute greatly to G·C to T·A mutations found at this mutation hot spot in human cancers, particularly HCC. We propose that 4-HNE−DNA adducts may play an important role in human carcinogenesis, especially HCC.

ACKNOWLEDGMENT

We thank Drs. O. Bhanot, C. B. Klein, and Yen-Yee Tang for their critical review of the manuscript.

REFERENCES

1. Esterbauer, H., Schaur, R. J., and Zollner, H. (1991) *Free Radical Biol. Med. 11*, 81−128.
2. Bartsch, H. (1999) in *Exocyclic DNA Adducts in Mutagenensis and Carcinogenesis* (Singer, B., and Bartsch, H., Eds.) pp 1−16, International Agency for Research on Cancer Scientific Publication 150, International Agency for Research on Cancer, Lyon, France.
3. Ames, B. N., and Gold, L. S. (1991) *Mutat. Res. 250*, 3−16.
4. Christians, F. C., Newcomb, T. G., and Loeb, L. A. (1995) *Prev. Med. 24*, 329−332.
5. Marnett, L. J., and Plastaras, J. P. (2001) *Trends Genet. 17*, 214−221.
6. Fazio, V. M., Rinaldi, M., Ciafre, S., Barrera, G., and Farace, M. G. (1993) *Mol. Aspects Med. 14*, 217−228.
7. Joel, S. K., Lalwani, N. D., and Reddy, J. K. (1986) *Cancer Res. 46*, 1324−1330.
8. Rushmore, T. H., Lim, Y. P., Farber, E., and Ghohal, A. K. (1984) *Cancer Lett. 24*, 251−255.
9. Shamberger, R. J. (1972) *J. Natl. Cancer Inst. 48*, 1491−1497.
10. Cerutti, P. A. (1985) *Science 277*, 375−381.
11. Bartsch, H., Hietanen, E., and Malaveille, C. (1989) *Free Radical Biol. Med. 7*, 637−644.
12. Trush, M. A., and Kensler, T. W. (1991) *Free Radical Biol. Med. 10*, 201−209.
13. Chung, F. L., Chen, H. J., and Nath, R. G. (1996) *Carcinogenesis 17*, 2105−2111.
14. Douki, T., and Ames, B. N. (1994) *Chem. Res. Toxicol. 7*, 511−518.
15. Yi, P., Zhan, D., Samokyszyh, V. M., Doerge, D. R., and Fu, P. P. (1997) *Chem. Res. Toxicol. 10*, 1259−1265.
16. Wacker, M., Schuler, D., Wanek, P., and Eder, E. (2000) *Chem. Res. Toxicol. 13*, 1165−1173.
17. Chung, F. L., Nath, R. G., Ocando, J., Nishikawa, A., and Zhang, L. (2000) *Cancer Res. 60*, 1507−1511.

18. Chung, F. L., Zhang, L., Ocando, J. E., and Nath, R. G. (1999) in *Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis* (Singer, B., and Bartsch, H., Eds.) pp 45−54, International Agency for Research on Cancer Scientific Publication 150, International Agency for Research on Cancer, Lyon, France.
19. Wacker, M., Wanek, P., and Eder, E. (2001) *Chem.-Biol. Interact. 137*, 269−283.
20. Hu, W., Feng, Z., Eveleigh, J., Iyer, G., Pan, J., Amin, S., Chung, F. L., and Tang, M.-s. (2002) *Carcinogenesis 23*, 1781−1789.
21. Hussain, S. P., Raja, K., Amstad, P. A., Sawyer, M., Trudel, L. J., Wogan, G. N., Hofseth, L. J., Shields, P. G., Billiar, T. R., Trautwein, C., Hohler, T., Galle, P. R., Phillips, D. H., Markin, R., Marrogi, A. J., and Harris, C. C. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 12770−12775.
22. Smela, M. E., Currier, S. S., Bailey, E. A., and Essigmann, J. M. (2001) *Carcinogenesis 22*, 535−545.
23. Ross, R. K., Yuan, J. M., Yu, M. C., Wogan, G. N., Qian, G. S., Tu, J. T., Groopman, J. D., Gao, Y. T., and Henderson, B. E. (1992) *Lancet 339*, 943−946.
24. Thorgeirsson, S. S., and Grisham, J. W. (2002) *Nat. Genet. 31*, 339−346.
25. Denissenko, M. F., Koudriakova, T. B., Smith, L., O'Connor, T. R., Riggs, A. D., and Pfeifer, G. P. (1998) *Oncogene 23*, 3007−3014.
26. Bannasch, P., Khoshkhou, N. I., Hacker, H. J., Radaeva, S., Mrozek, M., Zillmann, U., Kopp-Schneider, A., Haberkorn, U., Elgas, M., Tolle, T., Roggendorf, M., and Toshkov, I. (1995) *Cancer Res. 55*, 3318−3330.
27. Chung, F. L., Chen, H. J., Guttenplan, J. B., Nishikawa, A., and Hard, G. C. (1993) *Carcinogenesis 14*, 2073−2077.
28. Marnett, L. J., Hurd, H. K., Hollstein, M. C., Levin, D. E., Esterbauer, H., and Ames, B. N. (1985) *Mutat. Res. 148*, 25−34.
29. Tang, M.-s., Pierce, J. R., Doisy, R. P., Nazimiec, M. E., and MacLeod, M. C. (1992) *Biochemistry 31*, 8429−8436.
30. Tang, M.-s., Lieberman, M. W., and King, C. M. (1982) *Nature 299*, 646−648.
31. Canella, K. A., and Seidman, M. M. (2000) *Mutat. Res. 450*, 61−73.
32. Esterbauer, H., and Weger, W. (1967) *Monatsh. Chem. 78*, 1994−2000.
33. Sancar, A., and Rupp, W. D. (1983) *Cell 33*, 249−260.
34. Tang, M.-s. (1996) in *Technologies for Detection of DNA Damage and Mutations* (Pfeifer, G., Ed.) pp 139−153, Plenum Press, New York.
35. Lee, D. H., O'Connor, T. R., and Pfeifer, G. P. (2002) *Nucleic Acids Res. 30*, 3566−3573.
36. Yang, J. L., Maher, V. M., and McCormick, J. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3787−3791.
37. Nishikawa, A., Furukawa, F., Kasahara, K., Ikezaki, S., Itoh, T., Suzuki, T., Uchida, K., Kurihara, M., Hayashi, M., Miyata, N., and Hirose, M. (2000) *Cancer Lett. 148*, 81−86.
38. Yoon, J. H., Smith, L. E., Feng, Z., Tang, M.-s., Lee, C. S., and Pfeifer, G. P. (2001) *Cancer Res. 61*, 7110−7117.
39. Cheng, K. C., Preston, B. D., Cahill, D. S., Dosanjh, M. K., Singer, B., and Loeb, L. A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9974−9978.
40. Cajelli, E., Ferraris, A., and Brambilla, G. (1987) *Mutat. Res. 190*, 169−171.
41. Brambilla, G., Sciaba, L., Faggin, P., Maura, A., Marinari, U. M., Ferro, M., and Esterbauer, H. (1986) *Mutat. Res. 171*, 169−176.
42. Levin, D. E., Yamasaki, E., and Ames, B. N. (1982) *Mutat. Res. 94*, 315−330.
43. Isono, K., and Yourno, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1612−1617.
44. Kawanishi, M., Matsuda, T., Nakayama, A., Takebe, H., Matsui, S., and Yagi, T. (1998) *Mutat. Res. 417*, 65−73.
45. Kawanishi, M., Matsuda, T., Sasaki, G., Yagi, T., Matsui, S., and Takebe, H. (1998) *Carcinogenesis 19*, 69−72.
46. Greenblatt, M. S., Bennett, W. P., Hollstein, M., and Harris, C. C. (1994) *Cancer Res. 54*, 4855−4878.
47. Suzuki, H., Takahashi, T., Kuroishi, T., Suyama, M., Ariyoshi, Y., Takahashi, T., and Ueda, R. (1992) *Cancer Res. 52*, 734−736.
48. Lung, M. L., Chan, W. C., Zong, Y. S., Tang, C. M., Fok, C. L., Wong, K. T., Chan, L. K., and Lau, K. W. (1996) *Cancer Epidemiol., Biomarkers Prev. 5*, 277−284.
49. Buettner, V. L., Hill, K. A., Halangoda, A., and Sommer, S. S. (1999) *Environ. Mol. Mutagen. 33*, 320−324.
50. Dumenco, L., Oguey, D., Wu, J., Messier, N., and Fausto, N. (1995) *Hepatology 22*, 1279−1288.
51. Wang, X. W., Gibson, M. K., Vermeulen, W., Yeh, H., Forrester, K., Sturzbecher, H. W., Hoeijmakers, J. H. J., and Harris, C. C. (1995) *Cancer Res. 55*, 6012−6016.
52. Forrester, K., Lupold, S. E., Ott, V. L., Chay, C. H., Band, V., Wang, X. W., and Harris, C. C. (1995) *Oncogene 10*, 2103−2111.

BI034431G