Lack of Mutagenicity of Acrolein-Induced DNA Adducts in Mouse and Human Cells

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Abstract

Acrolein is an endogenous metabolite and a ubiquitous environmental pollutant. Recently, it has been suggested that acrolein is a major etiologic agent for tobacco smoking–related lung cancer. Despite the known DNA-damaging effects of acrolein, its mutagenicity to mammalian cells remains uncertain. We have investigated acrolein-induced DNA damage in relation to mutagenesis, with special focus on DNA repair, in mouse and human cells. We mapped the formation of acrolein-induced DNA adducts and the kinetics of repair of the induced lesions in the cII transgene, the mutational target, in acrolein-treated transgenic mouse fibroblasts. Acrolein-DNA adducts were formed preferentially at specific nucleotide positions, mainly at G:C base pairs, along the cII transgene. The induced acrolein-DNA adducts were moderately resistant to DNA repair. Quantification of cII mutant frequency in acrolein-treated cells, however, revealed that acrolein was not mutagenic to these cells at doses sufficient to produce DNA adducts. Determination of supF mutant frequency in DNA repair–proficient and DNA repair–deficient human fibroblasts transfected with acrolein-treated plasmids confirmed a lack of acrolein mutagenicity. Because CpG methylation may intensify acrolein-DNA adduction, we examined whether the extent of CpG methylation in the supF gene can determine acrolein-induced mutagenesis in human cells. Enhancement of acrolein-DNA adduction by methylating CpGs in the supF sequence did not elicit a mutagenic response in human fibroblasts, however. We conclude that acrolein is not mutagenic to mouse and human fibroblasts, regardless of DNA repair capacity or methylation status of CpGs, possibly because of a highly accurate replication bypass of the induced lesions.

Introduction

Acrolein is the structurally simplest member of the family of α,β-unsaturated aldehydes (enals; see Fig. 1; ref. 1). Human exposure to acrolein occurs through a wide range of sources, including environmental, occupational, medicinal, and natural sources (2, 3). Acrolein is a ubiquitous environmental pollutant, which is formed during combustion of organic materials, and as such can be found in all types of smoke (e.g., automotive exhaust, cooking oil fumes, and tobacco smoke; refs. 2, 4). As a prominent vapor-phase constituent of tobacco smoke, acrolein is formed at high concentrations in sidestream smoke (i.e., a moderate tar-filtered cigarette has an acrolein content of 1.2 mg in sidestream smoke versus 70 μg in mainstream smoke; ref. 5). Thus, environmental tobacco smoke imposes a major burden of acrolein on second-hand smokers (3). Acrolein is extensively used in the chemical industry, especially in the manufacturing of acrylic acid (2, 3). Annually, ~900 million pounds of acrolein is produced in the United States (6). Therapeutically, acrolein is a principal metabolite of the widely used anticancer drugs cyclophosphamide and ifosfamide (2, 3). Endogenously, acrolein is a metabolic end product of lipid peroxidation and a decomposition product of the cellular polyamine spermine (2, 3). The omnipresence of exogenous and endogenous acrolein makes human exposure to this chemical incessant and, to some extent, unavoidable.

Acrolein is the most reactive of all enals (1–3). The α,β-unsaturated carbon-carbon bond of acrolein can readily engage in Michael type addition with cellular nucleophiles to form alkylated adducts with glutathione, protein sulfhydryls, thiol-containing enzymes, and DNA (3). The reactivity of acrolein toward cellular DNA is of significance inasmuch as acrolein-induced DNA adducts may initiate mutagenesis, thereby being etiologically relevant for carcinogenesis.

Because of its extreme toxicity, acrolein has been difficult to characterize in standard animal carcinogenicity tests. According to an evaluation by the IARC, there is inadequate evidence for carcinogenicity of acrolein in humans or animals (4). An increased incidence of urinary bladder carcinomas was observed in rats receiving i.p. injections of acrolein in combination with uracil in the diet, although acrolein alone did not induce tumors (7). Thus, the available evidence for a carcinogenic potential of acrolein is weak.

Acrolein reacts directly with DNA, predominantly with guanine residues forming two pairs of stereoisomeric exocyclic propano adducts, including γ-hydroxypropano-2'-deoxyguanosine (γ-HOPdG) and α-hydroxypropano-2'-deoxyguanosine (α-HOPdG) adducts (see Fig. 1; refs. 8–10). Acrolein-induced DNA adducts have been characterized in vitro (11–14) and quantified in vivo in various organs of experimental animals and humans (9, 10, 15, 16). A recent in vitro study has shown preferential formation of acrolein-DNA adducts at lung cancer mutational hotspots in the p53 tumor suppressor gene in normal human bronchial epithelial cells and lung fibroblasts (17). Most recently, γ-HOPdG and α-HOPdG adducts have been detected in human lung DNA of current and ex-smokers, albeit independently of smoking status (18).

Acrolein-DNA adducts have displayed varying mutagenic potentials in different site-specific mutagenesis assays (19–26). In classic genetic toxicology tests, acrolein has also exhibited ambiguous mutagenicity (27–29). For example, acrolein has tested both positive (29) and negative (27, 30) in the Ames Salmonella assays using various tester strains. Investigations of acrolein mutagenicity to mammalian cells have been limited (31–35); however, the scant available data point to a possible role of DNA repair in acrolein-induced mutagenesis (32, 35). Using the 6-thioguanine resistance
assay, acrolein has been shown to be nonmutagenic to Chinese hamster ovary cells (AS52; ref. 34) and normal human fibroblasts (32), whereas significant mutagenic responses have been reported in V79 Chinese hamster cells deficient in the repair of O6-methylguanine (35) and in human fibroblasts derived from xeroderma pigmentosum complementation group A (XP1223) deficient in nucleotide excision repair (32).

To fill the gaps in knowledge of acrolein mutagenicity, we have conducted a series of comprehensive investigations to cover various aspects of acrolein-induced DNA damage/repair and mutagenesis. We have used a transgenic mouse model system, which is proved to be invaluable for correlation studies of DNA damage/repair and mutagenesis at both genomic and single nucleotide level (36). Here, we have determined the formation and kinetics of repair of acrolein-DNA adducts as well as analyzed the mutagenicity of the induced lesions in the cII transgene of Big Blue mouse embryonic fibroblasts. Having established the DNA-damaging and mutagenic properties of acrolein in mouse cells, we have extended our analysis to human fibroblasts both proficient and deficient in DNA repair using the supF mutagenesis assay (37–39).

Materials and Methods

Cell culture and chemical treatment. Of relevance for the present study, culturing of mouse embryonic fibroblasts under physiologic O2 tension (3%) minimizes the burden of oxidative stress on the cells and enhances their proliferation capacity (40). This leads to a significant reduction in the frequency of spontaneously derived mutations associated with in vitro aging and an accelerated accumulation of induced mutations (40, 41). Because under the physiologic O2 tension, mouse embryonic fibroblasts replicate approximately once every 24 h (40), we have determined the persistency of all acrolein-induced DNA lesions over 24 h posttreatment. Obviously, only in viable and replicating cells can the induced lesions be efficiently translated into mutations (36). Therefore, we initially examined acrolein cytotoxicity to mouse embryonic fibroblasts by establishing a kill curve for cells treated in vitro with increasing doses of this chemical (see Fig. 2). Briefly, early-passage Big Blue mouse embryonic fibroblasts were grown as monolayer at −30% to 40% confluence in DMEM supplemented with 10% fetal bovine serum. Before chemical treatment, the culture media were removed and the cells were washed thoroughly with PBS. The culture dishes were filled with serum-free DMEM and, subsequently, fresh acrolein (Sigma-Aldrich, Inc.) was added to the media at increasing concentrations of 0.00001 to 1 mmol/L. Incubation was done at 37°C for 6 h in the dark. Immediately after treatment or 24 h thereafter, the cells were harvested by trypsinization for evaluation of cytotoxicity and DNA damage/repair. Alternatively, the treated cells were cultured in complete growth medium for an additional 4 days, and afterward were subjected to mutation analysis of the cII transgene. The 4-day growing period is essential for the fixation of all mutations into the genome (36). At the time of harvesting, all cultured cells had undergone 3 to 4 population doublings. All experiments were conducted in triplicates. As positive control, parallel sets of cells were treated with potent mutagens, including benzo[a]pyrene diol epoxide (B[a]PDE) or UVB irradiation, according to our previously published protocols (42, 43). The latter cell cultures were processed alongside the acrolein-treated cultures throughout.

Genomic DNA isolation. To reduce the adventitious oxidation of DNA, we have used an optimized salt-based DNA isolation method as described earlier (43). Briefly, the harvested cells were washed with prewarmed PBS and lysed with 4 mL of a solution containing 0.5 mol/L Tris-HCl (pH 8.0), 1 mol/L NaCl, 0.5% sodium dodecyl sulfate, and 0.1 mol/L EDTA.

Figure 1. Chemical structures of acrolein-DNA adducts. The γ-HOPdG and α-HOPdG DNA adducts of acrolein are shown. The ring-open form of γ-HOPdG adduct is also depicted.
20 mmol/L EDTA (pH 8.0), 10 mmol/L NaCl, 1% SDS, and 0.5 mg/mL proteinase K at 37°C overnight. After addition of 2 ml of saturated NaCl (~6 M) to each sample, incubation was done at 65°C for 10 min. Subsequently, centrifugation was done at 5,000 g for 30 min, and the supernatant containing DNA was mixed with 2 volumes of prechilled absolute ethanol. The DNA was spilled by gently inverting the mix, washed with 70% ethanol, air-dried, and dissolved in Tris-EDTA buffer (1 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 7.5). All DNA samples were preserved at −80°C until further analysis.

**Terminal transferase-dependent PCR.** To evaluate DNA damage formation and repair, we have used terminal transferase-dependent PCR (TD-PCR) for footprinting of DNA adducts on the entire noncoding strand of the cII transgene (36). Methodologically, TD-PCR is based on the concept that DNA polymerase cannot synthesize DNA past certain types of lesions (e.g., polymerase blocking/pausing lesions; ref. 36). Both γ-HOPdG and α-HOPdG adducts of acrolein are known to block DNA polymerase (19, 24, 25). Thus, our TD-PCR analysis can indiscriminately detect both lesions (combined). The TD-PCR quantified adducts (i.e., γ-HOPdG and α-HOPdG adducts combined) are referred to as the “acrolein-DNA-adducts” from hereon. Briefly, genomic DNA was subjected to nine cycles of primer extension using a custom-made biotinylated primer (cII.P1,5-CCGCTCTTACACATTCCAGCCCTG-3; cII.P2,5-TAAATAACCCCGCTCTTAC-3) in a Vent (exo-) DNA polymerase (New England Biolabs). The thermocycler settings were as follows: 2 min at 95°C, 2 min at 65°C, and 3 min at 72°C; 45 s at 95°C, 2 min at 61°C, 3 min at 72°C; 21 cycles of 45 s at 95°C, 2 min at 61°C, 3 min at 72°C; 4 cycles of 45 s at 95°C, 2 min at 65°C, and 3 min at 72°C; 1 min at 95°C, 2 min at 60°C, and 10 min at 72°C. The labeled products were subjected to polyacrylamide-urea gel electrophoresis using an IR2 Long Ranger 4200 system with simultaneous detection (LI-COR; ref. 36).

**cII mutation analysis.** Genomic DNA of transgenic Big Blue rodents contains multiple copies of the chromosomally integrated XLIZ shuttle vector, which carries two reporter genes (i.e., the cII and lacI transgenes; ref. 45). The cII mutation detection system is based on the recovery of this coliphage vector from the genomic DNA, followed by a bacterial phenotypic expression assay (46). Briefly, the recovered vector is packaged into viable bacteriophages, and the infective phage particles are introduced into an indicator host Escherichia coli. The lambda phages can multiply either lytically or lysogenically in the host E. coli, depending on the status of cII transcription (47). The cII protein is indispensable for activating the cl repressor and lambda integrase, both of which are essential for lysogenization (47). The E. coli indicators that carry phages with a mutated cII undergo lysis, thereby forming visible plaques on special agar lawn (46). The lambda LIZ shuttle vector, however, harbors a cII857 temperature-sensitive (ts) mutation, which makes the cl(ts) protein labile at temperature exceeding 32°C (46). Consequently, all vector-bearing phages, irrespective of the status of cII mutation, multiply lytically in the host E. coli at incubating temperatures higher than 32°C (45). This temperature sensitivity is the basis for the cII selection assay in which phenotypic expression of the cII mutants is achieved under selective incubation condition (i.e., 24°C; ref. 46). Under nonselective incubation condition (i.e., 37°C), however, both wild-type and mutant cII are expressed (46). The ratio of plaques formed under the selective condition to those arisen under the nonselective condition is commonly referred to as the “cII mutant frequency,” which denotes the mutation rate in the cII transgene (36).

Briefly, the XLIZ shuttle vectors containing the cII transgene were recovered from the genomic DNA and packaged into viable phage particles using the Transpack Packaging Extract kit (Stratagene). After preadsorption of the phages to G1250 E. coli, the bacterial culture was grown on special TB1 agar plates. To select for cII mutants, the plates were incubated at 24°C for 48 h. Alternatively, the plates were incubated under nonselective condition (i.e., 37°C overnight) to express both the wild-type and mutant cII. Verification of all putative cII mutants was achieved by replating under the selective condition. To determine a statistically valid cII mutant frequency, a minimum of 3 × 10⁶ rescued phages were screened for each experimental condition, as recommended by the manufacturer (Stratagene).

**supF mutation analysis.** The pSP189 plasmid containing a randomly generated 8-bp signature sequence at the 3’-end of the E. coli tyrosine amber suppressor tRNA supF gene was a generous gift of Dr. M. Seidman.
(National Cancer Institute, Bethesda, MD). The mutagenesis assay is based on the ability of the supF gene to abolish the effects of an amber mutation in the lacZ gene of E. coli, which results in a lack of β-galactosidase activity (37). The supF gene enables read-through of a UAG stop codon in the amber mutated lacZ gene, thereby restoring a functional β-galactosidase (37). Methodologically, the supF-bearing pSP189 plasmid is used to transform a specific strain of bacteria, MBM7070, which carries the lacZ gene with the amber mutation (37–39). Subsequently, a phenotypic expression assay is done with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as the chromogenic substrate. The transformed bacteria with wild-type supF-bearing plasmids retain β-galactosidase activity and form blue colonies on an agar lawn. Conversely, colonies resulting from transformation of bacteria with mutant supF-bearing plasmids are white (37–39).

Of relevance for the present study, the supF assay offers the possibility of investigating how the status of CpG methylation can affect DNA adduct formation and mutagenesis. It has been shown that methylation of cytosines at CpG dinucleotides enhances the extent of acrolein binding to guanines in synthetically generated human p53 DNA fragments (17). Thus, we carried out a supplementary supF assay, in which the pSP189 plasmid was pretreated with CpG-specific DNA methylase that efficiently methylates all CpGs along the supF sequence (48). Briefly, the pSP189 plasmid was methylated in vitro using the SssI methylase (New England Biolabs) according to the manufacturer’s instructions. Control plasmid was mock methylated in the absence of S-adenosylmethionine, the methyl donor. Completion of methylation was confirmed by digestion of an aliquot of the reaction mixture with the methylation-sensitive restriction endonuclease HpaII (New England Biolabs). Methylated and unmethylated pSP189 plasmids were incubated with acrolein at increasing concentrations of 0.0001 to 1 mmol/L for 6 h at 37°C in the dark. The treated plasmids were then transfected into cultures of SV40-transformed DNA repair–deficient human XPA fibroblasts (GM4427) and the counterpart repair-proficient cells (GM637; American Type Culture Collection) using the LipofectAMINE 2000 kit (Invitrogen). The transfected cells were cultured for 72 h, and afterward were subjected to plasmid recovery using the QIAprep Spin Miniprep kit (Qiagen, Inc.). Unreplicated plasmids were removed by digestion with DpnI (New England Biolabs), which recognizes the bacterial adenine methylation pattern (48). The confirmed progeny plasmids were electroporated into MBM7070 bacteria, and the transformed bacteria were

![Figure 3. Mapping of acrolein-induced DNA adducts in the cII transgene. TD-PCR footprinting of the full-length cII transgene (noncoding strand) was done using the genomic DNA of mouse embryonic fibroblasts treated with acrolein in comparison with control. As positive control, genomic DNA of parallel sets of cells treated with B(a)PDE (1 μmol/L) or irradiated with UVB (2,760 J/m²) underwent the same analysis. A, DNA footprinting results from cells harvested immediately after acrolein treatment versus controls. B, DNA footprinting results from cells harvested 24 h after acrolein treatment versus control. For brevity, results at select doses are shown. M, molecular size marker; nt, nucleotide position.](image-url)
grown on agar plates containing 100 μg/ml ampicillin, 1 μmol/L isopropyl β-D-thiogalactoside, an inducer of β-galactosidase, and 100 μg/ml X-gal at 37°C overnight. Subsequently, wild-type (blue) and mutant (white) colonies were counted to determine the supF mutant frequency. As positive control, parallel sets of methylated and unmethylated plasmids were irradiated with UVB and processed alongside the acrolein-treated plasmids throughout.

**Statistical analysis.** Results are expressed as medians ± SD. All variables in differently treated versus control groups were compared using the Kruskal-Wallis one-way ANOVA for comparison of repeated measures in multiple groups. Comparison of all variables between two separate groups was done using the Wilcoxon signed-rank test for matched pair comparison of repeated measures. All statistical tests were two sided. *P* ≤ 0.05 was considered statistically significant.

**Results**

**Cytotoxicity examination.** Acrolein treatment of mouse embryonic fibroblasts caused concentration-dependent cytotoxicity, most visibly in the micromolar dose range, as determined by trypan blue dye exclusion assay (Fig. 2). At a dose of 100 μmol/L acrolein, cell survival was diminished to as low as 7.7 ± 6.5% (median ± SD). Doses of acrolein exceeding 100 μmol/L were absolutely cytotoxic. The obliterated cell viability at such concentrations of acrolein served as a guideline for dosing in DNA damage/repair and mutagenesis experiments. In the latter experiments, the choice of dosing is crucial for establishing viable and proliferative cells in which the induced DNA damage can be efficiently translated into mutations (36).

**DNA damage and repair in the cII transgene.** We used TD-PCR to map the formation and repair of DNA adducts in the cII transgene of acrolein-treated mouse embryonic fibroblasts (36). Under our experimental conditions, these cells undergo a full cell cycle approximately once every 24 h (40). Thus, we assessed the repair of DNA adducts in acrolein-treated cells, which retained a reasonable proliferative capacity, over 24 h posttreatment. The latter is of importance because persistent DNA adducts, which have not been removed from the genome before DNA replication, can engage in translesion DNA synthesis (TLS), thereby manifesting their mutagenic potentials (36). As shown in Fig. 3A, acrolein treatment of mouse embryonic fibroblasts induced DNA adducts at specific locations, mostly at G:C base pairs, along the cII transgene. The formation of DNA adducts was saturated at a dose of 10 μmol/L acrolein, most likely due to high cytotoxicity of acrolein at the higher doses. The preferential DNA adduction sites were as follows, nucleotide positions 26–29, 67–70, 89–90, 125–126, 135, 144–145, 160, 166, 270–271, and 277. The formation pattern of acrolein-DNA adducts was only partially similar to that of B[a]PDE-DNA adducts in the cII transgene. In contrast, the mapping of acrolein-DNA adducts was completely distinct from that of UVB-induced DNA adducts, the latter forming exclusively at dipyrimidine nucleotides along the cII transgene. For the most part, the induced DNA adducts were partly removed from the cII transgene 24 h posttreatment; however, appreciable adduct removal was detectable at nucleotide positions 125–126, 135, and 166 (Fig. 3B).

**cII mutation analysis.** Acrolein treatment of mouse embryonic fibroblasts at none of the tested doses was significantly mutagenic as it only marginally elevated the cII mutant frequency relative to background (see Table 1). The trivial increases in relative cII mutant frequency in cells treated with increasing concentrations of acrolein were not significantly different from one another, either. In contrast, both B[a]PDE treatment and UVB irradiation showed extreme mutagenicity to these cells by elevating the relative cII mutant frequencies ~26- and ~59-fold, respectively. Given the detectable formation of acrolein-DNA adducts in mouse embryonic fibroblasts, which were relatively persistent over a full cell cycle, the nonmutagenicity of acrolein to these cells can be ascribed to an accurate replication past these lesions.

**supF mutation analysis.** We determined acrolein mutagenicity to human cells using the supF mutagenesis approach (37), in which pSP189 plasmids carrying the mutational target supF gene were treated with acrolein and subsequently transfected into human fibroblasts. Because acrolein-DNA adduction is enhanced by the extent of CpG methylation (17), we treated both unmethylated and methylated plasmids with increasing doses of acrolein. There is precedence that acrolein mutagenicity might be modulated by DNA repair (32, 35); thus, we used both DNA repair–proficient and DNA repair–deficient cells for transfection. Overall, there was no significant increase in relative supF mutant frequency in either DNA repair–proficient or DNA repair–deficient human fibroblasts transfected with acrolein-treated unmethylated plasmids (see Tables 2 and 3). In all cases, the relative increase in supF mutant frequency in DNA repair–deficient human fibroblasts was slightly but nonsignificantly higher than those in counterpart DNA repair–proficient cells. Likewise, there was no significant increase in relative supF mutant frequency in either DNA repair–proficient or DNA repair–deficient human fibroblasts transfected with acrolein-treated methylated plasmids. However, there was a dose-dependent elevation of relative supF mutant frequency in UVB-irradiated unmethylated plasmids transfected into DNA repair–proficient human fibroblasts. An augmented dose-dependent increase in relative supF mutant frequency was observed in counterpart DNA repair–deficient cells transfected with UVB-irradiated unmethylated plasmids. In both cases, the extent of increases in relative supF mutant frequency was intensified in cells transfected with UVB-irradiated methylated plasmids.

**Discussion**

Acrolein is a ubiquitous environmental pollutant and an endogenously generated metabolic product, to which humans are

### Table 1. Mutant frequency of the cII transgene in Big Blue mouse embryonic fibroblasts treated with increasing concentrations of acrolein

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total no. plaques (pfu)</th>
<th>Mutant plaques</th>
<th>Mutant frequency (×10⁻⁵)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3,382,500</td>
<td>144</td>
<td>4.39 ± 0.65</td>
</tr>
<tr>
<td>Acrolein (10 nmol/L)</td>
<td>1,918,333</td>
<td>97</td>
<td>5.09 ± 0.82</td>
</tr>
<tr>
<td>Acrolein (100 nmol/L)</td>
<td>3,095,833</td>
<td>158</td>
<td>5.38 ± 0.72</td>
</tr>
<tr>
<td>Acrolein (1 μmol/L)</td>
<td>1,775,833</td>
<td>97</td>
<td>5.56 ± 0.44</td>
</tr>
<tr>
<td>Acrolein (10 μmol/L)</td>
<td>3,353,500</td>
<td>180</td>
<td>5.62 ± 0.60</td>
</tr>
<tr>
<td>Acrolein (25 μmol/L)</td>
<td>1,850,000</td>
<td>99</td>
<td>5.43 ± 0.99</td>
</tr>
<tr>
<td>Acrolein (50 μmol/L)</td>
<td>1,944,500</td>
<td>104</td>
<td>5.23 ± 0.69</td>
</tr>
<tr>
<td>Acrolein (100 μmol/L)</td>
<td>3,180,000</td>
<td>171</td>
<td>5.19 ± 0.43</td>
</tr>
<tr>
<td>B[a]PDE (200 nmol/L)</td>
<td>1,272,500</td>
<td>1,427</td>
<td>11.50 ± 6.72</td>
</tr>
<tr>
<td>UVB (2,760 J/m²)</td>
<td>1,115,000</td>
<td>2,963</td>
<td>260.50 ± 11.56</td>
</tr>
</tbody>
</table>

*Each treatment condition was assayed at least thrice and the results are expressed as median ± SD.

 Abbreviation: pfu, plaque-forming units.
the kinetics of repair of the induced lesions in the we mapped the formation of acrolein-induced DNA adducts and cells, as shown in Fig. 2. Having established a relevant dosing range, doses higher than 100 acrolein cytotoxicity to mouse embryonic fibroblasts. Acrolein at and mutagenesis at a physiologic level, we initially determined objective of our study to correlate DNA damage, kinetics of repair, induced DNA damage in relation to mutagenesis, with special focus unclear. In the present study, we have investigated acrolein-acrolein, its mutagenicity to mammalian cells has remained constantly exposed. Despite the known DNA-damaging effects of acrolein, its mutagenicity to mammalian cells has remained unclear. In the present study, we have investigated acrolein-DNA adducts were fairly persistent over a full cell cycle, nucleotide positions along the mutational target gene, in acrolein-treated mouse fibroblasts. Acrolein-DNA adducts were formed preferentially at specific nucleotide positions along the cII transgene (Fig. 3A). The induced acrolein-DNA adducts were fairly persistent over a full cell cycle, thus being of relevance for mutagenesis (Fig. 3B).

Using a similar experimental design to that of ours, Feng et al. (17) have recently identified the type of DNA adducts formed in cultured lung cells as well as in purified genomic DNA treated with acrolein with the 32P postlabeling method. The authors have shown that γ-HOPdG is the major acrolein-DNA adduct formed in both acrolein-treated cells and acrolein-modified genomic DNA (17). Using high concentrations of acrolein (1.1 mol/L) for modifications of calf thymus DNA, Chung et al. (7) detected α-HOPdG adducts; however, this type of adduct was not detectable in acrolein-treated Chinese hamster ovary cells, liver samples from mice and rats, and human liver and oral tissue samples (8–10). In contrast, detectable levels of γ-HOPdG adducts were observed in the same samples throughout (8–10). The low or lack of detectability of α-HOPdG adducts in various experimental systems might be ascribed to structural constraints in genomic DNA and chromatin structure, which could hinder the formation of such adducts (17).

Determination of cII mutant frequency in acrolein-treated mouse fibroblasts, however, revealed that acrolein was not mutagenic to these cells at doses sufficient to produce DNA adducts (Table 1). Conversely, B[a]PDE showed extreme mutagenicity to the same

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total no. colonies</th>
<th>No. mutant colonies</th>
<th>Mutant frequency (×10^{-3})*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UM pSP189 M pSP189</td>
<td>UM pSP189 M pSP189</td>
<td>UM pSP189 M pSP189</td>
</tr>
<tr>
<td>Control</td>
<td>44,649 51,085</td>
<td>29 55</td>
<td>0.67 ± 0.03 0.77 ± 0.67</td>
</tr>
<tr>
<td>Acrolein (0.1 μmol/L)</td>
<td>31,896 70,616</td>
<td>20 60</td>
<td>0.66 ± 0.09 0.83 ± 0.10</td>
</tr>
<tr>
<td>Acrolein (1 μmol/L)</td>
<td>61,360 68,372</td>
<td>48 61</td>
<td>0.73 ± 0.57 1.04 ± 0.33</td>
</tr>
<tr>
<td>Acrolein (10 μmol/L)</td>
<td>49,566 61,840</td>
<td>37 52</td>
<td>0.95 ± 0.24 0.94 ± 0.22</td>
</tr>
<tr>
<td>Acrolein (100 μmol/L)</td>
<td>54,949 39,897</td>
<td>61 39</td>
<td>0.83 ± 0.46 0.87 ± 0.38</td>
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<tr>
<td>Acrolein (1,000 μmol/L)</td>
<td>44,050 74,688</td>
<td>37 86</td>
<td>0.85 ± 0.10 1.22 ± 0.20</td>
</tr>
<tr>
<td>UVB (2,760 J/cm²)</td>
<td>31,008 43,864</td>
<td>41 144</td>
<td>1.25 ± 0.19 3.21 ± 0.43</td>
</tr>
<tr>
<td>UVB (5,520 J/cm²)</td>
<td>30,278 77,960</td>
<td>85 531</td>
<td>2.82 ± 0.40 6.78 ± 1.07</td>
</tr>
</tbody>
</table>

* Each treatment condition was assayed at least thrice and the results are expressed as median ± SD.
† Unmethylated pSP189 plasmid: mock methylation was done in the absence of S-adenosylmethionine.
‡ Methylated pSP189 plasmid: methylation was done with the SsoI methylase and S-adenosylmethionine.

<table>
<thead>
<tr>
<th>Treatment</th>
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<tr>
<td></td>
<td>UM pSP189 M pSP189</td>
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<td>UM pSP189 M pSP189</td>
</tr>
<tr>
<td>Control</td>
<td>19,304 29,062</td>
<td>9 16</td>
<td>0.43 ± 0.20 0.54 ± 0.03</td>
</tr>
<tr>
<td>Acrolein (0.1 μmol/L)</td>
<td>59,304 87,216</td>
<td>64 49</td>
<td>1.11 ± 0.71 0.59 ± 0.09</td>
</tr>
<tr>
<td>Acrolein (1 μmol/L)</td>
<td>51,720 59,016</td>
<td>62 49</td>
<td>1.03 ± 0.94 0.82 ± 0.08</td>
</tr>
<tr>
<td>Acrolein (10 μmol/L)</td>
<td>54,808 39,112</td>
<td>52 25</td>
<td>0.97 ± 0.59 0.56 ± 0.24</td>
</tr>
<tr>
<td>Acrolein (100 μmol/L)</td>
<td>45,984 69,624</td>
<td>57 45</td>
<td>1.14 ± 0.64 0.61 ± 0.22</td>
</tr>
<tr>
<td>Acrolein (1,000 μmol/L)</td>
<td>21,666 34,101</td>
<td>35 29</td>
<td>1.23 ± 1.29 0.72 ± 0.20</td>
</tr>
<tr>
<td>UVB (2,760 J/cm²)</td>
<td>33,920 48,349</td>
<td>252 543</td>
<td>7.23 ± 0.70 10.55 ± 1.39</td>
</tr>
<tr>
<td>UVB (5,520 J/cm²)</td>
<td>19,089 54,351</td>
<td>226 850</td>
<td>10.31 ± 3.35 15.68 ± 0.11</td>
</tr>
</tbody>
</table>

* Each treatment condition was assayed at least thrice and the results are expressed as median ± SD.
† Unmethylated pSP189 plasmid: mock methylation was done in the absence of S-adenosylmethionine.
‡ Methylated pSP189 plasmid: methylation was done with the SsoI methylase and S-adenosylmethionine.
cells while forming a partially similar pattern of DNA adducts to that formed by acrolein. This implies that unlike the highly miscoding B[a]PDE-DNA adducts, which undergo error-prone TLS, acrolein-DNA adducts are either poorly miscoding or subject to high-fidelity error-free DNA polymerase(s) bypass. Quantification of supF mutant frequency in human fibroblasts transfected with acrolein-treated plasmids verified a lack of acrolein mutagenicity to these cells, as well (see Tables 2 and 3). The nonmutagenicity of acrolein to human fibroblasts, regardless of their DNA repair capacity, reiterates the notion that in vivo acrolein-DNA adducts either do not miscode or are processed through a highly accurate TLS. Because CpG methylation is known to enhance acrolein-DNA adduction (17), we also examined whether the extent of CpG methylation in the supF gene can affect acrolein-induced mutagenesis in human cells (in the Big Blue system, all CpGs along the cll transgene are highly methylated; ref. 49). As shown in Tables 2 and 3, enhancement of acrolein-DNA adduction by methylating CpGs in the supF sequence did not elicite a mutagenic response in human fibroblasts. This reaffirms the notion that formation of acrolein-DNA adducts per se is not sufficient to induce mutagenesis.

The existing in vivo site-specific mutagenicity studies have consistently shown an efficient TLS mostly in an error-free manner across γ-HOPdG adduct (20, 22, 24, 25). Error-free lesion bypass has also been shown in vivo for α-HOPdG adduct (21, 25). In vitro, however, low-fidelity TLS has been reported for both of these acrolein-DNA adduct (19, 24, 25). Using an oligonucleotide containing a site-specific γ-HOPdG adduct incorporated into heteroduplex DNA and propagated in E. coli, Wang et al. (20) have shown that γ-HOPdG is not mutagenic in vivo due to accurate TLS, nucleotide excision repair, and recombinational repair. Using the same adduct in both single-stranded and double-stranded shuttle vectors introduced into E. coli, VanderVeen et al. (19) have shown that in vivo γ-HOPdG is not a strong block to DNA replication and does not miscode. Replication and mutation assays in simian kidney cells (COS-7) using a single stranded vector containing site-specific γ-HOPdG have shown that in vivo DNA synthesis past this lesion is not significantly inhibited and correct lesion bypass occurs ~93% of the time (24). Mostly accurate replication bypass has also been reported for both γ-HOPdG and α-HOPdG adducts using single-stranded modified vectors transfected into COS-7 cells and amplified in E. coli (i.e., correct incorporation rates for the respective lesions were 92.6% and 91.7%; ref. 25). Using a double-stranded shuttle vector containing a site-specific γ-HOPdG, Yang et al. (22) have shown ~73% to 77% efficient TLS across this lesion with ≤1% miscoding frequency in three different human cell lines including XPA, XPV, and HeLa cells. Applying a similar approach, ~17% efficiency and 87.5% to 89.6% fidelity in TLS have been reported for α-HOPdG in human XPA cells (21).

Conversely, in vitro replication and lesion bypass assays of γ-HOPdG with the Klenow fragment (exo-) of E. coli polymerase I have shown a strong blockage of DNA replication and a limited, yet mostly error-prone, DNA synthesis across this lesion (19). In addition, in vitro, γ-HOPdG has been shown to severely block DNA synthesis with both polymerase δ and polymerase ε, two major eukaryotic polymerases (50), as well as elicit inaccurate lesion bypass (19, 24). Comparative in vitro replication assays with γ-HOPdG– and α-HOPdG–modified oligodeoxynucleotides and yeast polymerase γ show appreciable replicative blockage on both added templates, with α-HOPdG-modified substrate causing stronger obstruction (25).

Altogether, the discrepant mutagenicity of acrolein-DNA adducts in vivo and in vitro has been ascribed to a lack of critical accessory factors in vitro that mediate the catalyzing activities of specialized DNA polymerases operating on acrolein-induced DNA adducts in vivo (21, 23). As to the processing of acrolein DNA-adducts, yeast studies have shown that Rev1 efficiently incorporates a C opposite γ-HOPdG from which polymerase ξ extends, thereby completing an accurate lesion bypass reaction (26). In addition, a nuclear magnetic resonance structural study (51) has shown that within the duplex DNA, the exocyclic ring of γ-HOPdG opens when paired to dC, thereby enabling the pairing bases to assume a conventional Watson-Crick conformation (anti-anti). This structural rearrangement of γ-HOPdG from the ring-closed to the ring-open form leaves the acrolein-derived moiety, which is linked through the N2 position of dG, lying in the minor groove (see Fig. 1).

In such conformation, the N2-propyl chain of the adducted moiety points away from the minor groove, thus causing no distortion in DNA (51). This mechanism is not, however, applicable to α-HOPdG in the duplex DNA. Nonetheless, even if the exocyclic ring of α-HOPdG could open, the acrolein moiety would remain attached to the N1 position of dG, thereby preventing the Watson-Crick type pairing with dC. Presumably, a polymerase switching mechanism occurs at replication forks containing α-HOPdG adducts, wherein the major replicative polymerases are replaced by the as yet unknown lesion bypass polymerase(s), which carry out error-free TLS across these lesions (21, 22).

Recently, Feng et al. (17) have reported an unusually high mutagenic response for acrolein in human lung fibroblasts transfected with supF containing plasmid. In our hands, however, such extreme mutagenic response could not be obtained. Unfortunately, the experimental procedure in this report was not described in detail (i.e., specification of treatment conditions, duration of incubation, dose response relationship, etc.); thus, we were unable to exactly reproduce their experiments. We even examined an order of magnitude higher dose of acrolein compared with the only dose tested by Feng et al. (17); yet, no mutagenic response was detectable. To our knowledge, although different cell types used in their study versus theirs might have contributed, at least partially, to the discrepancy in results, the exact reason for such diverging findings remains unclear.

In conclusion, we have shown a lack of mutagenicity of acrolein-DNA adducts in mouse and human fibroblasts. The nonmutagenicity of acrolein to human cells was independent of DNA repair, and enhancement of acrolein-DNA adduction by methylating CpGs did not cause a mutagenic response in these cells, either. The nonmutagenicity of acrolein to mouse and human cells might be ascribed to the highly accurate replication bypass of acrolein-induced DNA lesions, previously established in site-specific mutagenesis experiments (19, 20, 22, 23). Our overall findings favor the notion that DNA adduct formation, in and of itself, is not sufficient to produce mutagenesis in vivo. More specifically, our data on the lack of mutagenicity of acrolein do not support the hypothesis that acrolein is a major etiologic agent for cigarette smoke–related lung cancer (17).

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References


Lack of Mutagenicity of Acrolein-Induced DNA Adducts in Mouse and Human Cells

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