Malondialdehyde, a Product of Lipid Peroxidation, Is Mutagenic in Human Cells*

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Malondialdehyde (MDA) is an endogenous genotoxic product of enzymatic and oxygen radical-induced lipid peroxidation whose adducts are known to exist in DNA isolated from healthy human beings. To evaluate the mutagenic potential of MDA in human cells, we reacted MDA with pSP189 shuttle vector DNA and then transfected them into human fibroblasts for replication. MDA induced up to a 15-fold increase in mutation frequency in the supF reporter gene compared with untreated DNA. Sequence analysis revealed that the majority of MDA-induced mutations occurred at GC base pairs. The most frequent mutations were large insertions and deletions, but base pair substitutions were also detected. MDA-induced mutations were completely abolished when the adducted shuttle vector was replicated in cells lacking nucleotide excision repair. MDA induction of large deletions and the apparent requirement for nucleotide excision repair suggested the possible involvement of a DNA interstrand cross-link as a premutagenic lesion. Indeed, MDA formed interstrand cross-links in duplex plasmids and oligonucleotides. Substrates containing the sequence 5′-d(CG) were preferentially cross-linked, consistent with the observation of base pair substitutions in 5′-d(CG) sites in the MDA-induced mutation spectrum. These experiments provide biological and biochemical evidence for the existence of MDA-induced DNA interstrand cross-links that could result from endogenous oxidative stress and likely have potent biological effects.

Cancer results from the accumulation of multiple mutations in key growth regulatory genes (1). These genetic changes are a consequence of the inherent chemical instability of DNA under physiological conditions, errors made by the DNA replication and maintenance machinery, and replication of DNA bases that are chemically modified as a result of exposure to exogenous or endogenous genotoxins (2–5). Considerable evidence indicates that endogenous DNA damage contributes significantly to the causation of cancer (e.g. Ref. 6).

MDA‡ (Fig. 1) is a potentially important contributor to DNA damage and mutation that is produced endogenously via lipid peroxidation and prostaglandin biosynthesis (7). MDA is mutagenic in bacterial and mammalian cell assays, and it is carcinogenic in rats (8–12). In Salmonella typhimurium, MDA induces insertions and deletions as well as base substitutions (8, 13, 14). Replication of MDA-modified single-stranded M13 genomes in Escherichia coli causes G→T, A→G, and C→T mutations (15). These three types of mutations reflect the principal sites of DNA modification by MDA in vitro (Fig. 1) (16–19). The most abundant MDA adduct (20, 21), M,G, formed by reaction with guanine residues, is detected in a range of tissues from healthy human beings (22, 23). Replication of phage genomes containing a site-specifically positioned M,G lesion in E. coli causes a significant increase in mutation frequency compared with unadducted phage DNA (24). The mutation frequency is increased ~3-fold if the bacteria replicating the lesion are nucleotide excision repair (NER)-deficient (24). Thus the major MDA-DNA adduct, M,G, is 1) found in human tissues, 2) is mutagenic in bacteria, and 3) is a substrate for NER.

An important unanswered question is: is MDA mutagenic in human cells? To address this question, we employed a random mutagenesis approach that has been utilized successfully to evaluate the genotoxicity of a number of chemical agents (25). The double-stranded shuttle vector, pSP189, was reacted with MDA under physiological conditions and then transformed into human cells for replication. MDA was found to induce mutations in this system demonstrating for the first time that this metabolic product is a human mutagen. Subsequent studies suggested the involvement of a previously uncharacterized lesion, an MDA-DNA interstrand cross-link (ICL).

MATERIALS AND METHODS

Cells and Plasmids—The pSP189 shuttle vector (25), as well as the indicator bacteria, MBM7070, were provided by Michael Seidman (NIA, National Institutes of Health). The transformed human embryonic kidney cell line, Ad293 (American Type Culture Collection), was cultured in Eagle’s minimum essential medium supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), vitamins, and amino acids. The wild-type GM00683T and NER-deficient XP12Be (Coriell Cell Repository) and XP12RO (James Cleaver, University of California, San Francisco) human fibroblasts are SV40-transformed and express the large T antigen. Both NER-deficient cell lines were originally derived from patients with xeroderma pigmentosum complementation group A and have less than 2% of normal UV repair (28). All cell cultures were

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† The abbreviations used are: MDA, malondialdehyde; M,G, pyrimido[1,2-a]purin-10(3H)-one; NER, nucleotide excision repair; GC/
The products were neutralized with 2 mM K2CO3 and reacted with 5% methanol, dried, and then hydrolyzed to the base with 2.5% formic acid.

Comparison with known concentrations of authentic nucleoside standards. An aliquot of the digested material was examined by reversed phase 4B (Amersham Biosciences) (30). The M1GdR was eluted with phosphate buffer, pH 7.4. The concentration was determined spectrophotometrically (ε195 = 34,200 M⁻¹ cm⁻¹) (28). Varying amounts of MDA were reacted with pSP189 DNA (0.5 mg/ml in 125 mM sodium phosphate buffer, pH 7.4) for 24 h at 37 °C. DNA that was purified from soluble MDA using Centricron 3 centrifugal concentrators (Amicon), washed with 10 volumes of water, and concentrated by ethanol precipitation. A fraction of each pellet was examined by agarose gel electrophoresis to determine the extent of nicking caused by modification.

Quantification of M1G Levels—M1G levels were determined in MDA-treated plasmid DNA using gas chromatography/electron capture/negative chemical ionization/mass spectrometry (GC/ECN/ICMS) by a modification of procedures described previously (29). In brief, [2H2]M1G-deoxyribose (M1G-dR) internal standard was added to the modification of procedures described previously (29). In brief, DNA was hydrolyzed enzymatically and then purified of protein using an Ultrafree-Probind 0.45-

Detection of Mutations—Plasmid DNA was amplified treated plasmid DNA using gas chromatography/electron capture/negative chemical ionization/mass spectrometry (GC/ECN/ICMS) by a modification of procedures described previously (29). In brief, DNA was hydrolyzed enzymatically and then purified of protein using an Ultrafree-Probind 0.45-

Transfection and Rescue of pSP189—Human cells were transfected with modified or unmodified pSP189 by calcium phosphate coprecipitation (27). After 48 h, the cells were lysed, and the plasmid DNA was isolated by the Hirt method (31). The recovered DNA was digested with DpnI to remove unreplicated plasmid prior to transformation or by DpnI plus MboI to demonstrate completeness of DpnI digestion.

Characterization of the Mutations—Mutant colonies were picked, grown overnight in a 5-ml culture of LB containing ampicillin and tetracycline, and then restreaked twice on selective plates containing X-gal and IPTG to confirm their phenotype. Plasmid DNA was amplified and isolated by alkaline lysis. A fraction of each DNA preparation was linearized with EcoRI and analyzed by agarose gel electrophoresis for alterations in DNA mobility indicative of large insertions or deletions. Mutant DNA was sequenced by thermal cycling (Circumvent Sequencing Kit, New England Biolabs) using the primer oligonucleotide 5'-d(GGCGACACGGAAATGTTGAA), which was 5'-32P-end-labeled. Twenty cycles of amplification were required (94 °C for 1 min; 57 °C for 1 min; 72 °C for 1 min). Reactions were run on an 8% denaturing polyacrylamide gel (dPAGE, National Diagnostics) and visualized by autoradiography. Sibling mutants were identified as any pair of mutant DNAs that arose from the same MDA-modification reaction and human cell transfection that exhibited the identical mutations and signature sequences and scored only once in the mutational analysis (25).

Plasmid Assay for DNA ICLs—The assay was adapted from the procedure of Hartley et al. (32). In brief, plasmid pBR322 DNA (40 μg) was linearized with EcoRI, dephosphorylated with calf intestinal alkaline phosphatase, and then 5'-32P-end-labeled with T4 polynucleotide kinase. Enzyme and excess ATP were removed by phenol extraction and BioSpin-6 chromatography (BioRad), respectively. MDA was reacted with DNA (33,000 cpm) in MOPS buffer (125 mM, pH 7.0) for 24 h at 37 °C in a total volume of 25 μl. The samples were heat-denatured at 90 °C for 5 min in the presence of 2.5 μl of strand separation buffer (0.04% bromophenol blue, 0.04% xylene cyanol, 30% Me2SO, 1 mM EDTA) and then rapidly cooled on ice. The denatured single-stranded DNA was separated from non-denatured double-stranded DNA by agarose gel electrophoresis. The gel was dried, and radioactive bands were visualized by PhosphorImager analysis (Amersham Biosciences). Non-denatured linear pBR322 diluted in 15% Ficoll loading buffer was used as a marker of duplex DNA.

Oligonucleotide Assay for DNA ICLs—One hundred pmol of dPAGE-purified oligonucleotide 5'-d(CCAGCGAGATC) (Midland) was end-labeled with 32P and repurified as described above. The DNA was reacted under vacuum and then resuspended in 20 μl of annealing buffer (10 mM MOPS, pH 7.4 and 6 mM MgCl2) with an equimolar amount of the unlabeled complementary oligonucleotide. The oligonucleotides were annealed by heating at 90 °C for 5 min followed by a slow cool to room temperature. Aliquots of the duplex were modified with 150 μM Na1-MDA (in H2O) or 25 μM β-benzoyloxyacryl (BBA) (in Me2SO, synthesized as described previously (33)) in 125 mM sodium phosphate buffer, pH 7.4.

**Fig. 1. Structures of the most abundant adducts formed by reaction of MDA with DNA.** Reaction of DNA with the non-physiological structural analog of MDA, BBA, yields the same adducts, in the same proportion, albeit in greater yield (40).
buffer, pH 7.4, at 25 °C for 24 h in a total volume of 20 μl. Alternatively, aliquots of the duplex were modified for 3 h with 1 mM melphalan (in 0.1 N HCl, Sigma), 1 mM mechlorethamine (in 0.1 N HCl, Sigma), or for 1 h with 1 mM N-acetoxy-2-acetylaminofluorene (N-AAAP) (in ethanol, NCI Chemical Reference Standard Repositories). Reactions were stopped by the addition of loading dye and loaded directly onto a 20% dPAGE.

Results

MDA Modification of Plasmid DNA—Double-stranded pSP189 DNA was reacted with a range of concentrations of MDA at physiological temperature and pH (37 °C, pH 7.4, sodium phosphate buffer). Modification was evaluated by determining the levels of M1G in the shuttle vector DNA by GC/EC/NCI/MS. M1G concentrations increased linearly (R² = 0.99) with increasing amounts of MDA (Fig. 3A). The range of adduct levels detected corresponds to 0.01 to 0.1 mg G per shuttle vector in the samples used for mutagenesis.

Mutation Frequency Induced by MDA in Human Cells—Unmodified or MDA-modified pSP189 was transiently transfected into Ad293 human kidney cells. After 2–3 doubling times, the plasmid was recovered from the cells, transformed into an indicator bacterium, and screened for mutations in the supF reporter gene using a β-galactosidase α-complementation assay. The background mutation frequency in supF from unmodified pSP189 replicated in Ad293 cells was 1.3 × 10⁻⁶ which is comparable with published values (34, 35). MDA induced a dose-responsive increase in the mutation frequency ranging from 4.8 to 20 × 10⁻⁴ (Fig. 3B). The mutation frequency increased sharply at low concentrations of MDA (0.5–5 mM) and then plateaued or increased only slightly above 25 mM MDA to a maximum of 15-fold above background. At the lowest concentration of MDA tested, the mutation frequency was significantly greater than background (4.8 × 10⁻⁴ versus 1.3 × 10⁻⁴). Evaluation of lower concentrations of MDA (10 and 100 μM) revealed a 1.9- and 2.1-fold increase in mutation frequency, respectively (data not shown).

Several controls were performed to confirm that the mutations observed were a consequence of replication of MDA-induced DNA adducts in human cells. First, because nicks are mutagenic lesions (36), we confirmed by agarose gel electrophoresis that exposure of double-stranded pSP189 DNA to MDA did not induce nicking (data not shown). Second, pSP189 vector DNA recovered from the human cells was digested with DpnI prior to transformation of bacteria to remove all DNA that was not replicated by the human cells. Sequential digestion of rescued pSP189 with DpnI and MboI (the latter of which cuts only DNA replicated by mammalian cells) reduced the number of bacterial transformants by >99.5% indicating that DpnI digestion efficiently removed unreplicated DNA. Finally, MDA-modified pSP189 was transformed directly into MBM7070 bacterium and found to be non-mutagenic unless the bacteria were SOS-induced with UV irradiation prior to transformation (Fig. 3C). Because non-induced MBM7070 was used for detection of mutations in pSP189 harvested from human cells, this experiment demonstrated that the MDA-induced mutations were not generated in the bacteria during the screen.

Characterization of MDA-induced Mutations—A total of 12 spontaneous and 207 MDA-induced mutant plasmids were amplified, and the supF gene was sequenced. A fraction of each mutant plasmid was linearized by EcoRI digestion and analyzed by agarose gel electrophoresis for a shift in mobility indicative of a large insertion or deletion. One of the spontaneous mutants and 36% of the MDA-induced mutants exhibited such a mobility shift (data not shown). Six percent of the MDA-induced mutants carried multiple mutations within the supF gene. The occurrence of multiple mutations increased with the concentration of MDA used. The classes of mutations induced by MDA did not vary at different concentrations of MDA.

Four of the twelve spontaneous mutants sequenced contained a unique mutation within the 250 bp surrounding the 85-bp supF coding sequence (Table I). These included 1 insertion, 1 deletion, and 2 base substitutions. MDA induced predominantly insertions and deletions (63% of all mutations). Large deletions were most frequent (40% of all mutations); however, large insertions also were observed (16% of all mutations). Small insertions and deletions (≤2 bp) were less common; the most frequent were 2-base deletions (3%). Base substitutions comprised 37% of all MDA-induced mutations, the majority of which were at GC base pairs (92% of all substitutions). A similar number of transitions and transversions were observed.

Positioning of the MDA-induced base substitutions along the supF sequence (Fig. 4) demonstrated that the mutations were spread throughout the reporter gene. There was only one mutational hot spot (defined as >5% of observed mutations at a single base pair) at position 36. This hot spot was unusual in that all three of the possible base substitutions were observed with approximately equal frequency. Position 36 does not correlate with a previously reported hot spot for spontaneous mutations in the supF gene (37, 38), but it is a hot spot for UV (25) and ionizing radiation-induced mutations (39).
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TABLE I

Classification of mutations induced by MDA in supF

<table>
<thead>
<tr>
<th>Sequence change</th>
<th>Spontaneous mutations</th>
<th>MDA-induced mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insertion/deletion</td>
<td>2</td>
<td>131 (63%)^a</td>
</tr>
<tr>
<td>Insertion</td>
<td>1</td>
<td>38 (18%)</td>
</tr>
<tr>
<td>+1</td>
<td>0</td>
<td>4 (2%)</td>
</tr>
<tr>
<td>+2</td>
<td>0</td>
<td>1 (0.5%)</td>
</tr>
<tr>
<td>&gt;2b</td>
<td>1</td>
<td>33 (16%)</td>
</tr>
<tr>
<td>Deletion</td>
<td>1</td>
<td>93 (45%)</td>
</tr>
<tr>
<td>+1</td>
<td>0</td>
<td>3 (2%)</td>
</tr>
<tr>
<td>+2</td>
<td>0</td>
<td>7 (3%)</td>
</tr>
<tr>
<td>&gt;2b</td>
<td>1</td>
<td>83 (40%)</td>
</tr>
<tr>
<td>Base substitutions</td>
<td>2</td>
<td>76 (37%)</td>
</tr>
<tr>
<td>Transitions</td>
<td>1</td>
<td>37 (18%)</td>
</tr>
<tr>
<td>GC→AT</td>
<td>1</td>
<td>35 (17%)</td>
</tr>
<tr>
<td>AT→GC</td>
<td>0</td>
<td>2 (1%)</td>
</tr>
<tr>
<td>Transversions</td>
<td>1</td>
<td>39 (19%)</td>
</tr>
<tr>
<td>AT→TA</td>
<td>0</td>
<td>2 (1%)</td>
</tr>
<tr>
<td>AT→CG</td>
<td>0</td>
<td>2 (1%)</td>
</tr>
<tr>
<td>GC→TA</td>
<td>1</td>
<td>15 (7%)</td>
</tr>
<tr>
<td>GC→CG</td>
<td>0</td>
<td>20 (10%)</td>
</tr>
</tbody>
</table>

^a Percent of all MDA-induced mutations.

^b Where complete sequences were not obtained, the presence of large insertions and deletions was inferred from the mobility of the linearized vector DNA on agarose gel compared with linearized wt pSP189.

**Fig. 3.** Quantification of the effects of reacting double-stranded DNA with MDA. A, estimation of the extent of DNA modification by MDA under physiological conditions. pSP189 was reacted with MDA for 24 h at 37 °C in 125 mM sodium phosphate and then hydrolyzed and analyzed for one MDA adduct, M1G, by GC/EC/NCI/MS as described under “Materials and Methods.” The non-zero intercept represents artifactual noise in the mass spectrometer. This value was not subtracted from each datum point. B, mutation frequencies induced by modification of pSP189 with MDA prior to replication in human cells. The plasmid was reacted with MDA as described above and then transfected into wild-type (wt) human Ad293 or NER-deficient XP12Be cells. After replication, the vector DNA was reisolated and transformed into MBM7070 E. coli to screen for mutations in the supF reporter gene based on lacZ complementation. C, mutation frequencies induced by MDA-modified pSP189 when replicated in E. coli with or without SOS induction. MBM7070 cells were UV-irradiated to induce the SOS functions and then transformed with MDA-modified pSP189 by electroporation. Plating with X-gal and IPTG allowed screening for mutations in the supF reporter gene of the plasmid (as described under “Material and Methods”). The non-induced bacteria were handled identically with the exception of UV exposure.

**Mutation Frequency Induced by MDA in NER-deficient Fibroblasts**—Double-stranded DNA containing M1G is a substrate for bacterial NER (24). Thus, we investigated the ability of MDA-modified pSP189 to induce mutations in human cell lines derived from a patient with xeroderma pigmentosum complementation group A (XP-A). We anticipated that higher mutation frequencies would be observed in the NER-deficient cells compared with repair-competent cells. Contrary to expectations, replication of MDA-modified pSP189 resulted in a complete abrogation of mutagenesis (Fig. 3B).

To rule out the possibility that this result was due to a difference between the cell lines other than their DNA repair capacity (e.g. tissue source or mechanism of immortalization), the assay was repeated using another set of wild-type and XP-A-deficient fibroblast lines that are both SV40-transformed (26). The same results were obtained, i.e. MDA was unable to induce an increase in mutation frequency above background levels in the absence of NER (data not shown). To rule out the possibility that this unexpected result was due to a highly genotoxin contaminant in the synthetic MDA, a structural analog of MDA, BBA (Fig. 1), was also tested in the mutation assay. BBA is synthesized by a different mechanism than MDA (33), and thus it is unlikely to contain similar putative contaminants. BBA was ~8-fold more mutagenic than MDA (data not shown) consistent with its increased reactivity with DNA under neutral conditions (40). Similar to MDA, however, BBA-induced mutagenesis was NER-dependent.

**In Vitro Assay to Detect DNA ICLs**—Because a mutation spectrum dominated by large rearrangements and the dependence of mutagenesis on functional NER are both consistent with a DNA ICL as a premutagenic lesion, we tested the ability of MDA to form such lesions in vitro. First, linear end-labeled pBR322 plasmid was reacted with MDA under conditions that mimicked the modification of pSP189 DNA. After 24 h at 37 °C, the DNA was heat-denatured and analyzed by agarose gel electrophoresis for the presence of non-denaturable double-stranded DNA (Fig. 5A). Unreacted DNA was completely de-natured to single strands (Fig. 5A, 2nd lane), whereas treatment of the plasmid with 25 mM MDA rendered ~10% of the sample non-denaturable (Fig. 5A, 4th lane) consistent with the rapid renaturation characteristic of cross-linked DNA. At 50 mM MDA, virtually all of the DNA appeared to be cross-linked.

Similarly, a duplex 32P-end-labeled oligonucleotide was reacted with MDA in vitro under conditions identical to those used for plasmid modification. An 11-bp, GC-rich sequence was chosen in accord with the mutation spectrum of MDA in which there was a preponderance of mutations at GC base pairs. The
...sequences within this duplex were included in other duplexes that yielded no cross-linked product. Thus, the major BBA-induced cross-link is inferred to occur between two deoxyguanosine residues on the opposite strands of the sequence 5’-d(CG). A significantly less abundant product was observed for the duplex 5’-d(ATTAATTAGATTTAT), which most likely corresponds to a C→A cross-link.

**DISCUSSION**

Exposure of double-stranded DNA to the endogenous electrophile, MDA, led to covalent modification of the DNA and elevated levels of mutations when the DNA was replicated in mammalian cells (Fig. 3). These data provide the first evidence that MDA-DNA lesions are mutagenic in human cells. The maximum increase in mutation frequency observed after MDA treatment was 15-fold above background levels. This level of increase is comparable with the mutation frequencies induced by UV light and by another endogenous genotoxin, nitric oxide (37, 45).

Because MDA is an endogenous product, physiological conditions were used when MDA was reacted with DNA in order to approximate the endogenous spectrum of lesions. Reactions were carried out at 37 °C and pH 7.4 in sodium phosphate buffer. One parameter that was not optimized to mimic physiological conditions was the concentration of MDA used in the reactions. The reasons for this are 2-fold. First, endogenous concentrations of MDA are not precisely known, with reported values varying over 2 orders of magnitude (between 30 nM and 3 μM) (46–48). Second, because we are able to quantify accurately the most abundant MDA-DNA adduct, M₁G (25), we can use the concentration of this adduct in the plasmid DNA as an index of total DNA damage. Reaction of the double-stranded plasmid with millimolar concentrations of MDA resulted in levels of M₁G ranging from 1 to 10 M₁G adducts per 10⁶ bp (Fig. 3A). These levels are comparable with endogenous levels of M₁G (22, 23). Thus, concentrations of MDA required to achieve endogenous levels of MDA adducts cause a significant increase in mutation frequency when modified DNA is replicated in human cells.

Greater than 90% of the MDA-induced base pair substitutions occurred at GC base pairs (Table I), consistent with premutagenic lesions being guanine and/or cytidine adducts. A cytidine adduct, M₁C, is formed by the reaction of MDA with the nucleoside (19). However, the yields are so low that M₁C is not detectable in MDA-modified double-stranded DNA (49), making it unlikely that this adduct contributes substantially to the mutation spectrum of MDA-modified pSP189. The MDA-
guanine adduct, M₁G, detected in our modified plasmid, causes G→T transversions and G→A transitions when replicated in *E. coli* (24), which is consistent with the base pair substitutions observed in our assay. However, for several reasons, it appears unlikely that M₁G is the major MDA-induced premutagenic lesion in human cells.

First, the predominant type of mutation induced by MDA was large insertions and deletions, which are not detected in site-specific mutagenesis experiments with the M₁G adduct (23). Second, the levels of the M₁G adduct increased linearly with increasing concentrations of MDA (Fig. 3A), but the mutation frequency plateaued at the highest concentrations of MDA tested (Fig. 3B). If M₁G was the predominant premutagenic lesion, one would expect both curves to have similar slopes. Third, MDA-induced mutagenesis in human cells required NER (Fig. 3B) as was reported previously in *Salmonella typhimurium* (8) and *E. coli* (10). In contrast, M₁G is a substrate of NER, and its mutagenic potential increases in NER-defective cells (24).

An MDA-induced premutagenic lesion that is consistent with the above findings is a DNA ICL. Drugs that cross-link DNA induce large insertions in the pSP189 mutation assay, similar to MDA (41, 50). ICLs may also explain why the mutation frequency leveled off at higher concentrations of MDA. ICLs are extremely toxic lesions because they block DNA and RNA polymerization. Any plasmid that retains an unre-

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**Fig. 5. In vitro assays to detect MDA-induced DNA ICLs.**

**A,** cross-linking of plasmid DNA. Linear pBR322 DNA was phosphorylated by [γ³²P]ATP followed by reaction with increasing concentrations of MDA. The DNA was heat-denatured and subjected to agarose gel electrophoresis. DNA containing cross-links was detected as the band (in lanes 4 and 5) that co-migrates with non-denatured plasmid (lane 1). *ds,* double-stranded; *ss,* single-stranded. **B,** cross-linking of duplex oligonucleotides. A GC-rich 11-bp oligonucleotide was 5′-³²P-end-labeled, purified, and annealed to its complementary sequence. The duplexes were reacted with a variety of genotoxins as described under “Materials and Methods.” The reactions were quenched by the addition of dPAGE loading buffer and electrophoresed under denaturing conditions to separate non-covalently associated strands. Reaction of the duplex with melphalan and mechlorethamine served as positive controls for the formation of DNA ICLs (5th and 6th lanes, respectively). Reaction with N-AAA F was used to indicate the gel mobility of a single strand of the duplex modified with bulky adducts but not cross-linked (lane 2). Reaction of the duplex with MDA or BBA (lanes 3 and 4, respectively) yielded a product that comigrated with the melphalan cross-linked product. **C,** sequence specificity of BBA cross-linking. A series of 18-bp oligonucleotide duplexes was designed to contain all possible combinations of tandem base pairs that might be cross-linked by a three-carbon dialdehyde. The unique target sequence of each duplex is indicated below the gel. The strands were end-labeled, annealed, and either reacted with BBA (B lanes) or were mock-treated (C lanes). The reactions were analyzed for the presence of nondenaturable products by dPAGE. Cross-linked complexes were detected as slower migrating species that comigrated with the product of the reaction of a duplex with the cross-linking agent mechlorethamine (M lanes).
paried ICL is not replicated, so it is lost to detection in this assay. Finally, ICL-induced mutagenesis is reported to be NER-dependent in plasmid-based assays (51–53).

Repair of ICLs necessitates excision of the lesion from both strands of the DNA likely via an obligatory double strand break intermediate (54). Subsequently, the double strand break is repaired by homologous recombination. In a plasmid-based assay, mutations are scored in an epichromosomal vector containing prokaryotic sequences; thus it is unclear if undamaged homologous DNA is available to complete the repair process. Therefore in the presence, but not the absence, of NER ICLs might be partly excised or converted to a double strand break. These intermediates of repair would then be mutagenic, yielding deletions with high frequency. In the absence of NER, cross-links persist, block replication of the plasmid, and thereby render the plasmid undetectable in this assay.

MDA-induced DNA-ICLs are known to exist although they were reported to be formed only at acidic pH (55). We were able to detect DNA ICLs in MDA-modified plasmid and short duplex oligonucleotides under reaction conditions identical to those used for the mutation assay (Fig. 3, A and B). Cross-links were detected at neutral pH only if the modification reaction was done in the absence of additional primary amines, which are known to compete for reaction with MDA (27). This likely explains inconsistencies with prior results (28).

We sought to identify the favored sequence for an MDA-induced DNA ICL. We were unable to detect the cross-linked product if MDA was reacted with long oligonucleotide duplexes that contain residues with primary amines only at central positions (data not shown). This is most likely due to a low yield because DNA ICLs form preferentially near the ends of duplex DNA (56). Reaction of the same duplexes with the highly reactive structural analog of MDA, BBA, did result in cross-linking (Fig. 5C). Only duplexes containing the sequence 5′-d(CG), or to a much lesser extent 5′-d(TAGAT), were cross-linked by BBA. These data combined with the fact that MDA reacts with G > C residues suggest that the preferred MDA-induced DNA ICL involves two deoxyguanosine residues situated in a 3′–5′ orientation. The minor cross-linked product might be C-A or A-A or even within a single GC base pair.

It is notable that Harris and co-workers (57) synthesized a model for the MDA-DNA ICL in which a saturated trimethylene functionality is incorporated between the two deoxyguanosine residues of the duplex sequence 5′-d(CG) (Fig. 6). The same ICL with the opposite orientation (in the sequence 5′-d(GC)) could not be synthesized because of the conformational instability of the product. This is consistent with the major ICL product detected in our reaction of BBA with different target sequences. In vitro, the synthetic cross-linked duplex is a substrate for human NER (58). This is consistent with our hypothesis that an MDA-ICL of pSP189 is a substrate for NER in human cells.

Within the supF reporter gene of pSP189, there are six 5′-(CG) sequences (Fig. 4). Eighteen percent of the MDA-induced base pair substitutions occurred within or immediately adjacent to these sites. This is similar to the frequency with which site-specific psoralen ICLs induce local base pair substitutions in a plasmid-based assay (52, 53). GC→AT transitions and GC→TA transversions predominated, as is the case for a mitomycin C-ICL between guanine residues of the sequence 5′-(CG). Similar to the base pair substitutions, 15% of the MDA-induced insertions and deletions occurred within or immediately adjacent to a 5′-(CG) sequence. Thus, there is a correlation between the preferred cross-link sequence, structural data, and a subset of the observed mutation spectrum (57). MDA-induced mutations at sites other than 5′-(CG) may have arisen as a result of NER-dependent incision of sequences flanking the 5′-(CG) ICLs (59, 60), through the intermediacy of cross-links at other sequences, or from other MDA-DNA adducts that trigger mutations in an NER-dependent fashion.

One apparent conundrum remains: the M1G adduct, if situated in single-stranded DNA, is mutagenic when replicated in bacteria (23). Furthermore, the adduct is a substrate for NER. Yet we observed no increase in mutation frequency when pSP189 containing M1G was replicated in NER-deficient human cells. One possible explanation is that M1G is chemically unstable in duplex DNA, opening readily to a ring-opened form, which is more easily bypassed by polymerases in vitro and is less mutagenic in vivo (61). Alternatively, it could be that M1G is a substrate for NER and a second more efficient DNA repair mechanism in human cells. In that case, in the absence of NER, M1G is efficiently recognized and repaired, but in the presence of NER the two pathways compete. Indeed, M1G is recognized and repaired by the mismatch repair pathway in bacteria (62).

However, the most probable explanation is that the shuttle vector mutation assay was not sensitive enough to detect the contribution of M1G to the mutation frequency. At the highest concentration of MDA tested, the level of M1G was less than 0.1 adducts per plasmid. The supF reporter gene is only 2% of pSP189 DNA, indicating that <0.2% of the reporter genes contained an M1G adduct. Experiments in which a single M1G adduct is placed in double-stranded DNA and replicated in mammalian cells suggest that the mutagenic efficiency of the adduct is <2%. Thus the contribution of M1G to mutagenesis in wild-type cells is predicted to be 0.1 × 0.2 × 2% = 4 × 10⁻⁶. This is well below background levels in this assay.

Together, these data represent the first investigation of the biological consequences of the replication of MDA-modified double-stranded DNA in human cells. Not only does the study reveal that MDA-induced DNA damage is mutagenic, it also provides evidence for the occurrence of a previously undetected lesion that may be highly mutagenic, a DNA ICL. This lesion may contribute significantly to the genotoxicity associated with lipid peroxidation and oxidative stress.

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REFERENCES


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