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).

MDA1 (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), MgG, 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 MgG 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, MgG, 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 GM00637F and NER-deficient XP12Be (Coriel 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 (26). All cell cultures were

EC/NCI/MS, gas chromatography/electron capture/negative chemical ionization/mass spectrometry; ICL, interstrand cross-link; dPAGE, denaturing polyacrylamide gel electrophoresis; BBA, β-benzoyloxyacrolein; N-AAAFT, N-acetoxy-N-acetylaminofluorene; M1C, N2′-oxopropanyl-deoxycytidine; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; XP, xeroderma pigmentosum; IPTG, isopropyl-β-D-thiogalactoside; MOPS, 4-morpholinoethanesulfonic acid.

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‡ The abbreviations used are: MDA, malondialdehyde; M1G, pyrimido[1,2-a]purin-10(3H)-one; NER, nucleotide excision repair; GC/
found negative for mycoplasma by reverse transcriptase-PCR (Stratagene) two passages prior to experimentation.

Modification of pSP189 DNA—The pSP189 vector was amplified from a vector strain of E. coli, AB2463 (Genetic Stock Center, Yale University, New Haven, CT). Plasmid DNA was purified from the bacteria by alkaline lysis followed by cesium chloride banding (27). Na+–MDA was synthesized as described previously and dissolved in 125 mM sodium phosphate buffer, pH 7.4. The concentration was determined spectrophotometrically (εmax = 3.42 x 104 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. Then the DNA was purified from soluble MDA using Centricon 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 M, G Levels—M, G levels were determined in MDA-treated plasmid DNA using gas chromatography/electron capture/negative chemical ionization/mass spectrometry (GC/EC/NCI/MS) by a modification of procedures described previously (29). In brief, [2H2]M1G-deoxyribose (M1GdR) was eluted with an Ultrafree-Probind 0.45-m filter (Millipore). The M1G levels were determined in MDA-modified pSP189. The DNA was hydrolyzed enzymatically and then purified of protein using an Ultrafree-Probind 0.45-m filter (Millipore). An aliquot of the digested material was examined by reversed phase high pressure liquid chromatography to quantify the DNA by comparison with known concentrations of authentic nucleoside standards. The remainder of the sample was purified by immunoaffinity chromatography using a monoclonal antibody to M1G coupled to Sepharose 4B (Amersham Biosciences) (30). The M1GdR was eluted with methanol, dried, and then hydrolyzed to the base with 2.5% formic acid. The products were neutralized with 2 mM K2CO3 and reacted with 5% α-bromoo-2,3,4,5,6-pentafluorotoluene (Aldrich). The derivatized M1G was purified over 100 mg of silica (Supelclean LC-Si, Supelco), and the M1GdR was eluted with an Ultrafree-Probind 0.45-m filter (Millipore). Quantities of M1G were determined from the ratio of the heights of the M1G-PFB peak to the [2H2]M1G-PFB peak as related to a standard curve.

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 Mbol to demonstrate completeness of DpnI digestion.

Detection of Mutations—DNA recovered from the human cells was used to transform MBB7070 E. coli by electroporation (25 kV, 200 microfarads, 25 ohms, 0.2-cm electrode gap; Bio-Rad GenePulser) and selected on Luria-Bertani broth agar plates containing ampicillin (50 mg/ml). Mutations in the supF gene of pSP189 were screened as described above. The DNA was digested 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(CTCAGCATGCGCTATG) (Midland) was end-labeled with [32P] and repurified as described above. The DNA was dried 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 mM Na+-MDA (in H2O) or 25 mM β-benzoyloxyacrylactone (BBA) (in Me2SO, synthesized as described previously (33)) in 125 mM sodium phosphate buffer

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).
Fig. 2. The oligonucleotide duplexes synthesized. The target cross-link sequence is shown in boldface. The oligonucleotides were gel-purified, \(^{32}\)P-end-labeled, and annealed to their complements as described under "Experimental Procedures." The duplexes were reacted with MDA, BBA, or mechlorethamine and examined by denaturing PAGE, also as described under "Experimental Procedures."

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 mephalan (in 0.1 x HCl, Sigma), 1 mM methloethamine (in 0.1 x HCl, Sigma), or for 1 h with 1 mM \(N\)-acetoxy-2-acetylaminofluorene (\(N\)-AAAF) (in ethanol, NCI Chemical Reference Standard Repositories). Reactions were stopped by the addition of loading dye and loaded directly onto a 20% dPAGE.

Sequence Specificity of MDA-DNA ICLs—Six possible nucleoside pairs were screened for MDA cross-linking: G-G, C-C, A-A, G-C, G-A, and C-A in both the \(3' \rightarrow 5' \) and \(5' \rightarrow 3' \) orientation. The pairs were centered, for the most part uniquely, in a series of 10 synthetic oligonucleotide duplexes. The sequences flanking the target sequence centered, for the most part uniquely, in a series of 10 synthetic oligonucleotide duplexes. The sequences flanking the target sequence centered, for the most part uniquely, in a series of 10 synthetic oligonucleotide duplexes. The sequences flanking the target sequence centered, for the most part uniquely, in a series of 10 synthetic oligonucleotide duplexes.

**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 \(M_G \) in the shuttle vector DNA by GC/EC/NCI/MS. \(M_G \) concentrations increased linearly (\(R^2 = 0.99 \) with increasing amounts of MDA (Fig. 3A). The range of adduct levels detected corresponds to 0.01 to 0.10 \(M_G \) per phosphate group 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 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 \(\beta\)-galactosidase \(a\)-complementation assay. The background mutation frequency in \(supF \) from unmodified pSP189 replicated in Ad293 cells was \(1.3 \times 10^{-4} \) which is comparable with published values (34, 35). MDA induced a dose-responsive increase in the mutation frequency ranging from 4.8 to \(20 \times 10^{-4} \) (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 (\(4.8 \times 10^{-4} \) versus 1.3 \(\times 10^{-4} \)). 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 premutagenic 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 bacteria 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 \(55\text{-bp} \ supF \) 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, small insertions and deletions were also 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 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).
After replication, the vector DNA was reisolated and transformed into wild-type (wt) human Ad293 or NER-deficient XP12Be cells. The plasmid was reacted with MDA as described above and then transformation by MDA under physiological conditions. pSP189 was reacted with a DNA ICL as a premutagenic lesion, we tested the ability of MDA to form such lesions. 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 dehydroxylated and analyzed for one MDA adduct, M1G, by GC/EC/NCI/MS assay. BBA is synthesized by a different mechanism than 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 contaminations. 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.

**TABLE I**

<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%)</td>
</tr>
<tr>
<td>Insertion</td>
<td>1</td>
<td>38 (18%)</td>
</tr>
<tr>
<td>Insertion +1</td>
<td>0</td>
<td>4 (2%)</td>
</tr>
<tr>
<td>Insertion +2</td>
<td>0</td>
<td>1 (0.5%)</td>
</tr>
<tr>
<td>Insertion +2b</td>
<td>1</td>
<td>33 (16%)</td>
</tr>
<tr>
<td>Deletion</td>
<td>1</td>
<td>93 (45%)</td>
</tr>
<tr>
<td>Deletion +1</td>
<td>0</td>
<td>3 (2%)</td>
</tr>
<tr>
<td>Deletion +2</td>
<td>0</td>
<td>7 (3%)</td>
</tr>
<tr>
<td>Deletion +2b</td>
<td>1</td>
<td>83 (40%)</td>
</tr>
<tr>
<td>Base substitutions</td>
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<td>76 (37%)</td>
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<tr>
<td>Transitions</td>
<td>1</td>
<td>37 (18%)</td>
</tr>
<tr>
<td>GC→AT</td>
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<td>35 (17%)</td>
</tr>
<tr>
<td>AT→GC</td>
<td>0</td>
<td>2 (1%)</td>
</tr>
<tr>
<td>Transversions</td>
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<td>39 (19%)</td>
</tr>
<tr>
<td>AT→TA</td>
<td>0</td>
<td>2 (1%)</td>
</tr>
<tr>
<td>AT→CG</td>
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<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.

d Downloading from https://www.jbc.org/ by guest on August 16, 2017
both DNA strands in the retarded bands was confirmed in retarded in the gel (Fig. 5B, 1 and 7% of total label, respectively) that were significantly reacted with N-alkylguanine demethylating agents (Fig. 5B, 2nd lane). The reaction of the duplex oligonucleotide with Melphalan (41) and mechlorethamine (42) were used as positive controls for the formation of DNA ICLs and yielded bands (1 and 7% of total label, respectively) that were significantly retarded in the gel (Fig. 5B, 5th and 6th lanes). The presence of both DNA strands in the retarded bands was confirmed in separate incubations in which one strand or the other was labeled with 32P before annealing and modification (data not shown). Reaction of the duplex with N-AAAF resulted in several bands that migrated similarly to the 12–14-base oligonucleotide standards (Fig. 5B, 2nd lane). N-AAAF produces large hydrophobic adducts but does not cross-link DNA (43) and was used to characterize the migration pattern of labeled strands that were adducted but not cross-linked to their complementary strands. Reaction of the duplex oligonucleotide with MDA resulted in a band that comigrated in denaturing PAGE with the ICLs (Fig. 5B, 3rd lane). Reacting the duplex with BBA resulted in a similar band, albeit in greater yield (0.8% of the total label versus 0.05% for MDA). In addition, the BBA reaction caused a broadening of the band corresponding to the single-stranded labeled oligonucleotide, consistent with monoadDITION (Fig. 5B, 4th lane).

**Analysis of the Base Specificity of a BBA-DNA ICL**—In order to identify the DNA bases that are likely to comprise an MDA-induced DNA ICL, a series of radiolabeled, duplex oligonucleotides with different sequences was reacted with MDA or BBA and analyzed by dPAGE. In selecting the sequences to be tested, the assumption was made that an MDA cross-link, of only 3 carbons, could not span a distance greater than that between adjacent base pairs of duplex DNA. Given two adjacent base pairs, three possible adduction sites (at G, C, or A), and two structural orientations (3' to 5' and 5' to 3' (44)), 12 potential cross-link substrates were tested via a series of 10 18-bp duplex oligonucleotides (see “Materials and Methods”). These potential sites were centered in AT-rich sequences to minimize alternative modification sites. Reaction of the duplexes with MDA did not result in a detectable shift in electrophoretic mobility (data not shown). Two of the substrates reacted with BBA yielded a product that comigrated with the mechlorethamine-induced cross-link (Fig. 5C). The major band was seen for duplexes containing the unique target sequence 5'-d(TCG). This was the only duplex that contained two deoxyguanosine residues in adjacent base pairs but on opposite strands (i.e. 3' to 5' orientation). All other potential target 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(ATGCTAGGTTATTAG), which most likely corresponds to a C to 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 pre-mutagenic 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 and deletions 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-
paired 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 episomal 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′-d(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 transversions and GC→TA transversions predominated, as is the case for a mitomycin C-ICL between guanine residues of the sequence 5′-d(CG). Similar to the base pair substitutions, 15% of the MDA-induced insertions and deletions occurred within or immediately adjacent to a 5′-d(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′-d(CG) may have arisen as a result of NER-dependent incision of sequences flanking the 5′-d(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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