Quantification of oxidative single-base and intrastrand cross-link lesions in unmethylated and CpG-methylated DNA induced by Fenton-type reagents

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ABSTRACT

Methylation of cytosine at CpG sites in mammalian cells plays an important role in the epigenetic regulation of gene expression. Here, we assessed the formation of single-nucleobase lesions and intrastrand cross-link lesions (i.e. G[8-5]C, C[5-8]G, mC[5m-8]G, and G[8-5m]mC, where ‘mC’ represents 5-methylcytosine) in unmethylated and the corresponding CpG-methylated synthetic double-stranded DNA upon treatment with Fenton-type reagents [i.e. H2O2, ascorbate together with Cu(II) or Fe(II)]. Our results showed that the yields of oxidative single-nucleobase lesions were considerably higher than those of the intrastrand cross-link lesions. Although no significant differences were found for the yields of single-base lesions induced from cytosine and mC, the G[8-5m]mC cross-link was induced >10 times more efficiently than the G[8-5]C cross-link. In addition, the mC[5m-8]G was induced at a level that was >15 times less than G[8-5m]mC, whereas the corresponding C[5-8]G intrastrand cross-link lesion was not detectable. Moreover, Cu(II) is >10-fold as effective as Fe(II) in inducing oxidative DNA lesions. These results suggest that oxidative intrastrand cross-link lesions formed at methylated-CpG sites may account for the previously reported mCG→TT tandem double mutations induced by Fenton-type reagents.

INTRODUCTION

Reactive oxygen species (ROS) are produced endogenously, during normal aerobic metabolism and under various pathological conditions, and exogenously, such as upon exposure to UV light, ionizing radiation, environmental mutagens and carcinogens. DNA is susceptible to damage by ROS, and the accumulation of oxidative DNA lesions is associated with aging and a variety of human diseases, including cancer and neurodegeneration (1,2).

A large number of single-nucleobase lesions induced by ROS have been widely studied for their formation, mutagenesis and repair (2,3). Most common point mutations induced by ROS are C→T transitions, suggesting that modified cytosine derivatives are among the most abundant and mutagenic oxidative DNA lesions (4–6). In mammalian cells, cytosines at CpG sites are frequently methylated at the C5 carbon to form 5-methylcytosine (mC), which accounts for ~4% of all dC residues in humans (7,8). The methylated CpGs are mutational hot spots in human p53 tumor suppressor gene, and the most common mutation is mC→T transition (9).

Pfeifer and coworkers (10) recently observed unusual mCG→TT tandem mutations when CpG-methylated pSP189 plasmid was treated with Cu(II)/H2O2/ascorbate and replicated in nucleotide excision repair (NER)-deficient human XPA cells, suggesting that vicinal base damages or intrastrand cross-link lesions formed at methylated CpG dinucleotide sites might be involved. In this context, we previously identified the mC[5m-8]G and G[8-5m]mC cross-link lesions (structures shown in Scheme 1), where the methyl carbon of mC and the C8 of its adjacent guanine are covalently bonded, in oligodeoxynucleotides (ODNs) upon exposure to γ rays under aerobic and anaerobic conditions (11,12). Along this line, C[5-8]G and G[8-5]C (Scheme 1) cross-link lesions, where the C5 carbon of cytosine is coupled with the C8 carbon of its neighboring guanine, can also be induced in aqueous solutions of synthetic ODNs exposed to γ- or X-rays (13,14).

Others (14–17) and we (11–13,18,19) demonstrated that intrastrand cross-link lesions could be initiated from a single radical of pyrimidine bases. However, the same radical is also able to couple readily with molecular
oxygen and the resulting peroxyl radical can be transformed to give single-nucleobase lesions. For instance, 5-formyl-2'-deoxycytidine (5-FmdC) and 5-hydroxymethyl-2'-deoxycytidine (5-HmdC) can form from the methyl radical of 5-methyl-2'-deoxycytidine (Scheme 2). Likewise, 5-hydroxy-2'-deoxycytidine (5-OHdC) can be induced from the 6-hydroxy-5-yl radical of 2'-deoxycytidine (Scheme 3). The quantification of intrastrand cross-link and single-nucleobase lesions initiated from the aforementioned radicals under aerobic conditions can allow for the assessment of the relative contributions of these two pathways, i.e. coupling with \( \text{O}_2 \) or with its vicinal guanine base. Furthermore, it is important to compare the profiles of oxidative lesions formed in methylated and unmethylated sequences, which may offer insights into understanding the ubiquitous \( \text{C} \rightarrow \text{T} \) and the unusual \( \text{mCG} \rightarrow \text{TT} \) mutations found at CpG sites (9,10).

Hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) is produced by endogenous metabolic processes, and it can lead to the formation of hydroxyl radical in the presence of transition metal ions in their reduced states, e.g. Fe(II) or Cu(I). In this context, copper binds to DNA with high affinity to G:C base pairs (20), and it plays a pivotal role in maintaining the structure and integrity of chromatin (21). Cu(II) and \( \text{H}_2\text{O}_2 \), often with the addition of ascorbic acid, can result in the formation of highly reactive species and produce extensive strand breaks (22) as well as many types of single-base lesions in DNA (23), which was found to be enhanced while DNA was packed into nucleosomes (24). It was proposed that free copper ion primarily mediates the formation of frank strand breaks, whereas
DNA-bound copper induces mainly nucleobase modifications through the formation of DNA–Cu(I)–H$_2$O$_2$ complexes (22,25).

Iron is another biologically relevant transition metal. In vitro studies showed that Fe(III)-dependent DNA fragmentation is much less extensive than that produced by equivalent amount of Cu(II) ions under otherwise comparable reaction conditions (26), and Fe(II) induces significantly less nucleobase damage in the presence of H$_2$O$_2$ than does Cu(II) (23,27). On the other hand, studies with Jurkat cells revealed that intracellular iron, but not copper, plays an important role in H$_2$O$_2$-mediated formation of strand breaks in DNA (28).

Although the structures of various single-nucleobase lesions induced by Fenton reactions have been well established, there were few reports on the formation of cross-linked nucleobase lesions from treatment with Fenton-type reagents. In the latter respect, Randerath and others (29–34) detected, by $^{32}$P-postlabeling assay, several bulky DNA adducts that are induced endogenously in animal tissues or from the Fenton reaction mixture of synthetic ODNs. On the grounds that some of the bulky adducts were commonly formed in ODNs housing-specific dinucleotide sequences, these authors proposed that these adducts might be intrastrand cross-link lesions (32). These bulky lesions were also found in tissues from animals exposed to pro-oxidant chemicals, which firmly linked the formation of the bulky adducts to ROS (31,35). More recently, Randerath et al. (36) demonstrated that some of the bulky DNA adducts actually contained the 8,5'-cyclo-2'-deoxyadenosine (cyclo-dA). The cyclo-dA bears a covalent bond between the C8 of adenine and the C5 carbon in the same nucleoside. There were also indications that intrastrand cross-links might be induced in salmon sperm DNA upon treatment with Fenton-type reagents; the structures of these putative cross-link lesions, however, remain elusive (37,38). Recently, we first reported the formation of a structurally defined G[8-5m]T intrastrand cross-link lesion, which bears a covalent bond between the methyl carbon of thymine and the C8 of its adjacent 5' guanine, in calf thymus DNA upon treatment with Cu(II)/H$_2$O$_2$/ascorbate (39). It remains to be established whether intrastrand cross-link lesions can also form between guanine and its neighboring cytosine or mC in DNA upon exposure to Fenton-type reagents.

In the present study, we synthesized several isotope-labeled oxidative single-base and cross-link lesions originated from 2'-deoxycytidine (dC) and 5-methyl-2'-deoxycytidine (5-mdC) (Scheme 1), and employed LC-MS/MS with the standard isotope dilution method to identify and quantify the single-nucleobase and intrastrand cross-link lesions formed in synthetic duplex ODNs upon incubation with Cu(II)/H$_2$O$_2$/ascorbate or Fe(II)/H$_2$O$_2$/ascorbate. The results allowed us to compare the reactivities of methylated and unmethylated cytosine residues toward Fenton-type reagents mediated by two different transition metal ions, i.e. iron and copper. Moreover, we demonstrated that G[8-5]C, mC[5m-8]G and G[8-5]mC cross-link lesions, which were previously shown to be generated upon exposure to γ rays (11–13), could also be induced by Fenton-type reagents.

Scheme 3. Generation of single-base and cross-link lesions from 6-hydroxy-5-yl radical of 2'-deoxycytidine.
with 15N, 13C and deuterium, respectively. The labeled nitrogen, carbon and hydrogen atoms were replaced incorporation are indicated by bold italic font, in which we quantified in this study. The sites of isotope
Scheme 1 depicts the structures of the lesions that earlier (39).

EXPERIMENTAL PROCEDURES

Materials

CuCl₂, (NH₄)₂Fe(SO₄)₂·6H₂O, l-methionine, l-ascorbic acid and alkaline phosphatase were from Sigma–Aldrich (St Louis, MO, USA). Hydrogen peroxide (30%) and nuclease P1 were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and MP Biomedicals (Aurora, OH, USA), respectively. Snake venom phosphodiesterase and calf spleen phosphodiesterase were obtained from US Biological (Swampscott, MA, USA). Common reagents for solid-phase DNA synthesis were obtained from Glen Research Co. (Sterling, VA, USA). Unmodified ODNs (Table 1) used in this study were purchased from Integrated DNA Technologies (Coralville, IA, USA) and purified by HPLC. [2-amino-1,3,7,9–15N₅]-8-oxo-2'-deoxyguanosine was synthesized as described previously (39).

Synthesis and characterization of compounds

Scheme 1 depicts the structures of the lesions that we quantified in this study. The sites of isotope incorporation are indicated by bold italic font, in which the desired radical precursor 5-(phenylthiomethyl)-2'-dC.

<table>
<thead>
<tr>
<th>ODNs</th>
<th>Sequences⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODN1/2</td>
<td>5'-XGXXGXXGXXGXXG-3'</td>
</tr>
<tr>
<td>ODN3/4</td>
<td>5'-TXGATGXXATGXXG-3'</td>
</tr>
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</table>

⁴ODN1/ODN3, X = C; ODN2/ODN4, X = mC.

To quantify the standard DNA lesions

The concentrations of stock solutions of 5-FmdC, 5-HmdC, 5-OHdC, 8-oxodG, d(G[8-5m]mC) and d(G[8-5]C) were determined by UV absorbance measurements. The molar extinction coefficients (in l/mol/cm) used to quantify the standard lesions were: 5-FmdC, ɛ₅₀₃₅ = 13 700; 5-HmdC, ɛ₅₀₃₅ = 9070; 5-OHdC, ɛ₅₀₃₅ = 6025 (4); 8-oxodG, ɛ₅₀₃₅ = 9700 (41); d(G[8-5m]mC), ɛ₅₀₃₅ = 23 800 and d(G[8-5]C), ɛ₅₀₃₅ = 22 800 (42). The ɛ values for 5-FmdC, 5-HmdC and d(G[8-5m]mC) were determined by ¹H-NMR (Figures S4–S6) following the previously described method (43).

Treatment of synthetic ODNs with Fenton-type reagents

The ODNs were annealed in a buffer containing 50 mM NaCl and 20 mM phosphate (pH 6.9) by heating the solution to 90°C and cooling slowly to room temperature. Aliquots of ODNs (5 nmol) were incubated with CuCl₂ or (NH₄)₂Fe(SO₄)₂ (6.25–100 μM), H₂O₂ (50–800 μM) and ascorbate (0.5–8 mM) in a 100-μl solution containing 5 mM NaCl and 50 mM phosphate (pH 7.0) at room temperature for 20–25 min with continuous argon bubbling. The solution was dried in a Speed-Vac and reconstituted in water for digestion with a combination of four enzymes (vide infra), and the digestion mixture was separated by HPLC to obtain the isotope-labeled d(G[8-5m]mC) (Figures S1 and S2). Dinucleoside monophosphate d(XG) was irradiated under the similar conditions as described above, and the irradiation mixture was separated by HPLC to render the isotope-labeled d(mC[5m-8]G).

Quantification of standard DNA lesions

The concentrations of stock solutions of 5-FmdC, 5-HmdC, 5-OHdC, 8-oxodG, d(G[8-5m]mC) and d(G[8-5]C) were determined by UV absorbance measurements. The molar extinction coefficients (in l/mol/cm) used to quantify the standard lesions were: 5-FmdC, ɛ₅₀₃₅ = 13 700; 5-HmdC, ɛ₅₀₃₅ = 9070; 5-OHdC, ɛ₅₀₃₅ = 6025 (4); 8-oxodG, ɛ₅₀₃₅ = 9700 (41); d(G[8-5m]mC), ɛ₅₀₃₅ = 23 800 and d(G[8-5]C), ɛ₅₀₃₅ = 22 800 (42). The ɛ values for 5-FmdC, 5-HmdC and d(G[8-5m]mC) were determined by ¹H-NMR (Figures S4–S6) following the previously described method (43).

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<table>
<thead>
<tr>
<th>Control</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
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<tbody>
<tr>
<td>Cu(II)/Fe(II) (μM)</td>
<td>100</td>
<td>6.75</td>
<td>12.5</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>H₂O₂ (μM)</td>
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<td>50</td>
<td>100</td>
<td>200</td>
<td>400</td>
</tr>
<tr>
<td>Ascorbate (mM)</td>
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<td>0.5</td>
<td>1.0</td>
<td>2.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

*All reactions were carried out in a 100-μl solution containing 5 nmol ODNs in 25 mM NaCl and 50 mM phosphate (pH 7.0).
Control experiments were also carried out to examine the effect of individual components in Fenton-type system on the formation of DNA lesions. First, we incubated ODNs with 200 μM H₂O₂ and 2 mM ascorbate in the absence of Cu(II) or Fe(II). Second, ODNs were incubated with 25 μM CuCl₂ or (NH₄)₂Fe(SO₄)₂ and 200 μM H₂O₂, and no ascorbate was added. In addition, experiments were carried out in the presence of 4 mM dimethyl sulfoxide (DMSO), a hydroxyl radical scavenger, for Fenton reaction condition C (Table 2).

**Enzymatic digestion of DNA**

Four units of nuclease P1, 0.005 unit of calf spleen phosphodiesterase, and 1.5 μl solution containing 300 mM sodium acetate (pH 5.0) and 10 mM zinc acetate were added to 15 μl ODN samples treated with Fenton-type reagent, and the digestion was carried out at 37°C for 6 h. To the digestion mixture were then added 10 μl of alkaline phosphatase, 0.05 μl of snake venom phosphodiesterase, and 5 μl of 0.5 M Tris–HCl buffer (pH 8.9). The digestion was continued at 37°C for 6 h, and the enzymes were removed by passing through a 10-kDa cutoff Centricon membrane (Millipore, Billerica, MA). The amount of nucleosides in the mixture was quantified by UV absorbance measurements, and to the mixture were then added isotope-labeled standard lesions. The resulting aliquots were subsequently subjected to HPLC enrichment and LC-MS/MS analysis.

**HPLC**

Off-line HPLC separation was performed on a system composed of a Hitachi L-6200A pump (Hitachi Ltd, Tokyo, Japan), an HP-1050 UV detector (Agilent Technologies, Palo Alto, CA, USA) and a Peak Simple Chromatography Data System (SRI Instruments Inc., Las Vegas, NV, USA). A 4.6 × 250 mm Apollo C18 column (5 μm in particle size, 300 A˚ in pore size, Alltech Associates Inc., Deerfield, IL, USA) was used for the separation of synthetic ODNs and the enzymatic digestion products of the UV irradiation mixture of the d(CCGXCCGG) and d(XG). The flow rate was 0.8 ml/min. For the ODN separation, a solution of 50 mM triethylammonium acetate (TEAA, pH 6.6, solution A) and a mixture of 50 mM TEAA and acetonitrile (70/30, v/v) (solution B) were used as mobile phases. A gradient of 5 min 0–20% B, 45 min 20–50% B and 5 min 50–100% B was employed. For the ODNs separation, a 60-min gradient of 0–25% acetonitrile in 10 mM ammonium acetate (pH 6.3) was employed, and the flow rate was 0.60 ml/min. We collected fractions in a wide retention time range (3–4 min) to ensure that the lesions were completely collected while the unmodified nucleosides were excluded as much as possible. The collected fractions were dried in a Speed-vac, and re-dissolved in 10 μl of H₂O for LC-MS/MS analysis.

**LC-MS/MS analysis**

On-line HPLC–MS/MS measurements were carried out using an Agilent 1100 capillary HPLC pump (Agilent Technologies) and an LTQ linear ion-trap mass spectrometer (Thermo Fisher Scientific), which was set up for monitoring the fragmentation of the [M + H]⁺ ions of the labeled and unlabeled single-base or cross-link lesions. A 0.5 × 150 mm Zorbax SB-C18 column (particle size 5 μm, Agilent Technologies) was used for the separation of the DNA hydrolysis samples, and the flow rate was 6.0 μl/min. A 60-min gradient of 0–25% acetonitrile in 20 mM ammonium acetate was employed for the analysis of HPLC-enriched oxidative lesions.

**RESULTS**

**LC-MS/MS identification and quantification of DNA lesions formed in ODNs 1 and 2 induced by Cu(II)/H₂O₂/ascorbate**

We annealed separately the self-complementary ODNs 1 and 2 (Table 1) to form duplexes and treated them with different concentrations of Cu(II)/H₂O₂/ascorbate (Table 2), digested the resulting DNA samples with enzymes, and analyzed the digestion mixtures by LC-MS/MS with stable isotope-labeled lesions as internal standards.

The selected-ion chromatogram (SIC) for the m/z 569 → 275 transition, which monitors the loss of 2-deoxyribose/phosphate backbone, showed a fraction eluting at the same time as the labeled d(G[8-5m]mC) internal standard (Figure 1a and b; the sample was from ODN2 treated under condition C listed in Table 2). Similarly, the SIC for m/z 569 → 275 transition, which showed a peak at the same retention time as the labeled d(mC[5m-8]G) at around 13 min, supported the formation of d(mC[5m-8]G) (Figure 1c and d). The product-ion spectrum of the ion of m/z 569 further revealed the presence of d(G[8-5m]mC and d(mC[5m-8]G) in the enzymatic digestion mixture of the treated ODNs (Figure 1). Moreover, the amounts of d(G[8-5m]mC) and d(mC[5m-8]G) are significantly lower in the control samples without hydrogen peroxide treatment, showing that the G[8-5m]mC and G[8-5m]mC intrastrand cross-link lesions can indeed be induced by Fenton-type reagents. In this regard, our previous studies revealed that G[8-5m]mC and C[5m-8]G could be initiated from the methyl group of mC (Schemes 1 and 2) (44).
Furthermore, the relative abundances of the two major fragment ions formed from the cleavage of the ion of m/z 555 match those formed from the fragmentation of the [M + H]+ ion of the internal standard, further supporting the presence of d(G[8-5]C) in the enzymatic digestion mixture of ODN1 after treatment with Fenton-type reagents (Figure 2). It is worth noting that we attempted, but failed to detect the d(C[5-8]G) in the enzymatic digestion mixture. The formation of G[8-5m]mC, mC[5m-8]G and G[8-5]C intrastrand cross-link lesions exhibited linear dose-dependent increase when the concentrations of H2O2 were up to 800 μM; however, the yield of G[8-5m]mC is 13 and 16 times greater than those of G[8-5]C and mC[5m-8]G, respectively, upon treatment with the same dose of Cu(II)/H2O2/ascorbate (Figure 3, and calibration curves for LC-MS/MS quantification are shown in Figure S12).

Previously we demonstrated that the yields of mC[5m-8]G and G[8-5m]mC are much higher under anaerobic than aerobic conditions, suggesting the competition between coupling with molecular oxygen and conjugating with its neighboring base (12). Given that OH can also result in the formation of single-base lesions under the same oxidation conditions, it is important to examine the formation of these lesions. Not surprisingly, LC-MS/MS analysis also confirmed the formation of 5-FmdC and 5-HmdC in ODN2 (Figures S7 and S8) and 5-OHdC in ODN1 upon treatment with Fenton-type reagents (Figure S9). The quantification results showed that, at doses up to 800 μM H2O2 and 100 μM Cu(II) (Table 2), the yields of the lesions increased proportionally with the rise in the concentrations of the Fenton-type reagents. Moreover, the yields of single-base lesions were 2–3 orders of magnitude higher than those of the cross-link lesions (Figure 3).
Iron is another biologically important transition metal which can participate in Fenton-type reactions. To assess the different effects of iron and copper on inducing oxidative DNA lesions, we also examined DNA lesions produced in ODNs 1 and 2 upon treatment with Fe(II)/H₂O₂/ascorbate. It turned out that the yields of cross-link and single-base lesions were markedly lower when Cu(II) was replaced with Fe(II) under otherwise identical experimental conditions (Figures 3 and 4). In addition, the amounts of d(mC[5m-8]G), d(C[5-8]G) and d(G[8-5]C) induced in ODNs 1 and 2 were below the detection limits of our LC-MS/MS method. Furthermore, the yields of 5-HmdC, 5-FmdC and d(G[8-5m]mC) rise proportionally with the increase of the concentration of Fenton-type reagents; however, the amount of 5-OHdC exhibited a slightly different trend. The yields of 5-OHdC at low-dose range increased linearly with the rise in the concentrations of Fenton-type reagents. On the other hand, when the DNA was treated with the highest dose of Fe(II)/H₂O₂/ascorbate ([H₂O₂] = 800 µM), the yield of 5-OHdC increased considerably, which was 10-fold higher than the yield found at the second highest dose ([H₂O₂] = 400 µM, Figure 4C). The exact reason for the disproportionally high yield found at the highest dose is not clear, though we suspect that the duplex DNA may undergo a conformational change at this high concentration of Fe(II).

**LC-MS/MS quantification of DNA lesions formed in ODNs 3 and 4 oxidized with Cu(II)/H₂O₂/ascorbate**

It is known that the methylation in poly(GC) sequence can facilitate the conformational change of DNA, most notably the transition from normal B to Z-form DNA in the presence of divalent metal ions (45). Therefore, we also treated two mixed-sequence ODNs (ODNs 3 and 4 in Table 1), which contain both the GX and XG sites (X = C or mC), with Cu(II)/H₂O₂/ascorbate at the two highest doses. It turned out that the yields of oxidative lesions are comparable with what we found for ODN1 and...
ODN2 (Figure 5). In addition, we quantified the yield of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG, Figures S10 and S11), an abundant and well-studied oxidative DNA lesion. Our results showed that the amount of 5-HmdC, 5-FmdC or 5-OHdC was 4–6-fold lower than that of 8-oxodG (Figure 5a and b).

Effects of individual components in Fenton-type reagents and a radical scavenger DMSO

We also carried out several control experiments to gain insights into the mechanism for the formation of the
oxidative lesions under Fenton-type reaction conditions. In the absence of transition metal ions, the yields of lesions were sharply reduced (Figure 6). Leaving out ascorbate resulted in pronounced decrease in the yields of both single-base and intrastrand cross-link lesions (Figure 6). However, treatment of duplex DNA with Fe(II)/H2O2 results in the formation of higher levels of lesions than the corresponding treatment with Cu(II)/H2O2 (Figure 6). This observation is in keeping with the fact that Fe(II) can react directly with H2O2 to generate -OH, whereas Cu(II) has to be reduced by ascorbate to give Cu(I) before it can participate in Fenton-type reaction to afford -OH.

We also incubated ODNs with Fenton-type reagents in the presence of DMSO, a free -OH scavenger, and assessed the formation of oxidative DNA lesions under these conditions. It turned out that the presence of DMSO does not result in obvious decrease in the yields of single-base and cross-link lesions. This result suggests that the DNA-bound metal ions induce site-specific formation of -OH, which cannot be intercepted by the radical quencher DMSO. The resulting nucleobase radical can then couple with either molecular oxygen and its neighboring nucleobase to form single-base and intrastrand cross-link lesions, respectively.

**DISCUSSION**

Here we demonstrated, by using LC-MS/MS with the standard isotope dilution method, that the treatment of duplex ODNs with Cu(II)/H2O2/ascorbate can lead to the formation of G[8-5]C, mC[5m-8]G and G[8-5m]mC. Quantification results revealed that the yields of G[8-5]C and mC[5m-8]G cross-links (Figure 3a and b) are comparable with what we reported for another intrastrand cross-link, G[8-5m]T, in calf thymus DNA treated with the similar doses of Cu(II)/H2O2/ascorbate (39). In contrast, the G[5-8]G lesion was below the detection limit of our LC-MS/MS method. The yield of G[8-5m]mC formed in methylated duplex ODNs is, however, 13-fold greater than that of G[8-5]C produced in unmethylated ODNs under the same oxidation conditions (Figure 3a and b). Together, these data support that, upon treatment with Fenton-type reagents, the formation of intrastrand cross-link between guanine and its neighboring mC occurs at a much greater efficiency than that between guanine and its neighboring unmethylated cytosine. Our results also revealed that Cu(II)/H2O2/ascorbate system is ~10 times as effective as Fe(II)/H2O2/ascorbate system in inducing these cross-link lesions.

Previous studies demonstrated that G[8-5]C, G[8-5m]T and G[8-5m]mC cross-link lesions reduced the stability of duplex DNA (46,47), and the former two lesions blocked DNA synthesis by replicative DNA polymerases (46,48). Moreover, G[8-5]C could be bypassed by yeast pol η, a translesion synthesis DNA polymerase, with reduced efficiency and fidelity of nucleotide incorporation at the site opposite the 5’ guanine moiety of the lesion (13). Recent *in vitro* repair studies revealed that both G[8-5]C and G[8-5m]mC could be recognized and incised by *Escherichia coli* UvrABC nucleases (47), suggesting that oxidative intrastrand lesions might be substrates for NER enzymes *in vivo*. Furthermore, we found that G[8-5m]mC-bearing substrates could be incised by UvrABC nuclease with lower efficiency than the
vector was treated with Cu(II)/H2O2/ascorbate and lower frequency when the corresponding unmethylated!

The corresponding CG shuttle vector was replicated in NER-deficient XPA cells. 

abstract a hydrogen atom from the methyl group of mC

lesion from the mC-bearing strand was not quantified in 

standards of thymidine glycol, the formation of this 

double bond of mC can lead to the formation of mC 

undergo facile dehydration to yield 5-OHdC or undergo 

to the C5=C6 double bond in cytosine, which can lead to 

the formation of cytosine glycol. Cytosine glycol can 

to give the methyl radical of the pyrimidine base, which

can further transform to give 5-HmdC and 5-FmdC under aerobic conditions (54). With the above quantifi-

cation results, it is reasonable to speculate that the total amount of hydroxyl radical-induced major single-

base lesions from mC can be several fold higher than 

those formed from unmethylated cytosine. The more 

facile formation of single-base lesion at mC than at 

unmethylated cytosine may account for the prevalent 

C→T transition mutation found at CpG site in human 
p53 gene, and mutation in p53 is a hallmark for many 
types of human tumors (55). In this context, both 5-

OHdC and 5-FmdC were found to be mutagenic in vivo; 
a mutation frequency of 0.05% was reported for the 
former after 5-OHdC-bearing single-stranded M13 

gene was propagated in E. coli cells (5), and a mutation frequency of 0.03–0.28% was found for the 
latter after the 5-FmdC-carrying double-stranded shuttle 
veectors were replicated in simian COS-7 cells (56). 
To our knowledge, the mutagenic properties of 5-HmdC 
have not yet been examined.

The methylation pattern of cytosine residues at CpG 
sites and post-translational modifications of histones are 
crucial for maintaining the epigenetic code in human cells 
(57). The modification at CpG sequence can result in 
disturbed gene regulation and heritable epigenetic changes 
in chromatin. Recent studies revealed that the presence of 
8-oxodG or 5-HmdC at CpG site reduced significantly the 
binding of DNA to methyl-CpG-binding proteins by at 
least 10-fold (58). These lesions also alter the site 
selectivity of cytosine methylation at CpG site induced 
by human maintenance DNA methyltransferase DNMT1 
(59,60). High yield of 8-oxodG, 5-HmdC and 5-FmdC 
observed in our experiment (Figures 3–5) may indicate 
that DNA damage-mediated alteration in methylation 
pattern and subsequent binding by methyl-CpG-binding 
proteins can be significant in vivo.

The intracellular concentrations of iron and copper ions 
can be dramatically increased under oxidative stress 
conditions through their release from the iron- or 
copper-storage proteins (61,62). In humans, genetic 
hemochromatosis and Wilson’s disease cause abnormal 
accumulation of iron and copper, respectively, in various 
organs. An accumulation of highly mutagenic oxidative 
lesions in genomic DNA has been considered to be 
relevant to iron-induced carcinogenesis in iron-overload 
diseases (63,64). Bulky DNA lesions were found in the 
liver of patients with Wilson’s disease and primary 
hemochromatosis (65). Our current results support the 
argument that transition metal ions, especially copper, 
can induce significant amount of DNA damage, including 
bulky intrastrand cross-link lesions, in the presence of 
antioxidant such as ascorbate, which reduces the high-
valence Cu2+/Fe3+ so as to generate hydroxyl radical via 
the Fenton-type reaction.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.
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