Oxidative damage to 5-methylcytosine in DNA

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ABSTRACT

Exposure of pyrimidines of DNA to ionizing radiation under aerobic conditions or oxidizing agents results in attack on the 5,6 double bond of the pyrimidine ring or on the exocyclic 5-methyl group. The primary product of oxidation of the 5,6 double bond of thymine is thymine glycol, while oxidation of the 5-methyl group yields 5-hydroxymethyluracil. Oxidation of the 5,6 double bond of cytosine yields cytosine glycol, which decomposes to 5-hydroxycytosine, 5-hydroxyuracil and uracil glycol, all of which are repaired in DNA by *Escherichia coli* endonuclease III. We now describe the products of oxidation of 5-methylcytosine in DNA. Poly(dG-[³H]dmC) was γ -irradiated or oxidized with hydrogen peroxide in the presence of Fe³⁺ and ascorbic acid. The oxidized co-polymer was incubated with endonuclease III or 5-hydroxymethyluracil-DNA glycosylase, to determine whether repairable products were formed, or digested to 2'-deoxyribonucleosides, to determine the total complement of oxidative products. Oxidative attack on 5-methylcytosine resulted primarily in formation of thymine glycol. The radio-genic yield of thymine glycol in poly(dG-dmC) was the same as that in poly(dA-dT), demonstrating that 5-methylcytosine residues in DNA were equally susceptible to radiation-induced oxidation as were thymine residues.

INTRODUCTION

Oxidative damage to cellular DNA may result from the action of reactive oxygen species, such as peroxides, superoxide and hydroxyl radicals, all of which are produced during normal cellular oxidative metabolism (1). Recent studies have provided evidence for the role of oxidative damage to DNA in the pathology of Parkinson's disease and amyotrophic lateral sclerosis. It has also been suggested that accumulation of oxidative DNA damage is involved in cellular aging (2,3).

Reactive oxygen species produce a broad spectrum of DNA damage, including single strand breaks, damage to the deoxyribose moiety and damage to the purine and pyrimidine bases (4,5). Oxidative attack on the 5,6 double bond of pyrimidines results primarily in glycol formation. Thymine glycol is chemically stable and acts primarily as a block to replication. It is not pre-mutagenic,

because, when by-passed by polymerase, it pairs with adenine (6). In contrast, cytosine glycol is unstable, undergoing decomposition via dehydration and/or deamination to 5-hydroxycytosine, 5-hydroxyuracil or uracil glycol. 5-Hydroxycytosine has already been shown to be mutagenic in a phage system (7). 5-Hydroxyuracil and uracil glycol are both pre-mutagenic lesions, since they would pair with adenine, resulting in GC→AT transitions. All of these oxidized derivatives are substrates for *Escherichia coli* endonuclease III (8).

While thymine and cytosine constitute the bulk of pyrimidines in the DNA of most species, 5-methylcytosine may comprise up to 5% of all cytosine residues in mammalian genomic DNA. Methylation of cytosine occurs post-DNA synthesis, primarily in CpG doublets. It is thought that methylation of cytosine is involved in regulation of gene expression and recently it has been demonstrated that methylation suppresses the mitogenic properties of DNA incubated *in vitro* with B cells (9). 5-Methylcytosine residues in DNA are also 'hot spots' for mutation. 5-Methylcytosine to thymine transition mutations occur ~10 times as frequently as cytosine to thymine transitions at other sites (10). Such 5-methylcytosine mutations are frequently seen in heritable human diseases and inactivating mutations in the *p53* gene of tumors commonly occur in methylated CpG doublets.

This high degree of mutagenicity is attributed to the increased tendency of 5-methylcytosine to deaminate, as compared with cytosine, and the apparently relatively inefficient repair of the resulting GT mismatch. Since saturation of the 5,6 double bond of cytosine promotes deamination (11), we sought to determine what the products of oxidative attack on 5-methylcytosine residues in DNA were and to what extent they would lead to deamination. We used DNA methylase to transfer [³H]methyl groups from *S*-adenosyl-L-methionine to cytosine residues of poly(dG-dC) *in vitro*. Using this radioactively labeled DNA as a substrate and analytical HPLC as an assay system we characterized the formation of oxidative stress-induced damage to 5-methylcytosine in DNA and determined its enzymatic repairability using DNA glycosylases as reagent enzymes.

MATERIALS AND METHODS

Enzymes, DNA polymers and chemicals

SssI methylase (CpG methylase) was purchased from New England Biolabs. *Escherichia coli* endonuclease III was purified from *E. coli* λ N99 (CI857) containing the *nth* gene (12) and stored

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at -20°C at a concentration of 1 mg/ml. 5-Hydroxymethyluracil-DNA glycosylase (hmUDG) was purified from calf thymus as previously described (13). Alternating co-polymers poly(dG-dC) and poly(dA-dT) were purchased from Pharmacia. S -[methyl- ^3H]adenosyl-L-methionine (55–85 Ci/mmol) and [methyl- ^3H]thymidine-5'-triphosphate (70–90 Ci/mmol) were purchased from Du Pont.

Marker compounds

UV standard marker compounds 5-methylcytosine, thymine and 2'-deoxy-5-methylcytidine were purchased from Sigma. [^{14}C]Thymine glycol and [^{14}C]thymidine glycol were synthesized as previously described (14,15).

Synthesis of radiolabeled alternating DNA co-polymers

Poly(dG-[^3H]dmC) was synthesized from alternating poly(dG-dC) co-polymer by incubating with SssI methylase and S -[methyl- ^3H]adenosyl-L-methionine. Each reaction contained 250 μCi S -[^3H]adenosyl-L-methionine and 60 μg alternating co-polymer poly(dG-dC). Reactions were incubated overnight at 37°C . The DNA was separated from unincorporated radioactive material by ethanol precipitation, followed by passage through a Sephadex G-50 minicolumn. The purified DNA co-polymer was dissolved in 10 mM sodium phosphate buffer, pH 7.5.

Poly(dA-[^3H]dT) was synthesized from poly(dA-dT) by nick translation. Each translation reaction was conducted using 10 μCi [^3H]dTTP and 0.5 μg poly(dA-dT), *E. coli* DNA polymerase I and DNase I. Reaction mixtures were incubated for 2 h at 15°C and stopped by addition of 5 μl 0.25 M EDTA, followed by heating for 15 min at 70°C . Nick-translated DNA was separated from unincorporated radioactive nucleotide via a Sephadex G-50 column, followed by ethanol precipitation. The precipitated, purified DNA co-polymer was resuspended in 10 mM sodium phosphate buffer, pH 7.5.

γ -Irradiation of poly(dG-[^3H]dmC) and poly(dA-[^3H]dT)

Poly(dG-[^3H]dmC) or poly(dA-[^3H]dT) were passed through Sephadex G-50 minicolumns equilibrated with double glass-distilled water prior to irradiation at room temperature by a Gammator ^{137}Cs source to 100, 200, 300 and 400 Gy. After irradiation DNA samples were purified by passage through Sephadex G-50 columns equilibrated with 100 mM KCl, 50 mM Tris, 1 mM EDTA, pH 7.5 (TKE buffer) to remove small DNA fragments or the damaged bases released from the DNA backbone resulting from γ -irradiation. The TKE buffer was used in the *in vitro* endonuclease III assay.

Oxidation of poly(dG-[^3H]dmC) with H_2O_2 in the presence of iron

Poly(dG-[^3H]dmC) in double distilled water was incubated with 2.8 mM H_2O_2 , 50 μM FeCl_3 and 200 μM ascorbic acid (16) at 37°C for 60 min. After reaction the DNA sample was purified by passage through Sephadex G-50 columns equilibrated with TKE to remove oxidizing agents and DNA fragments.

Enzymatic assay of oxidized ^3H -containing co-polymers for release of oxidized pyrimidines

The purified oxidized co-polymer was incubated with endonuclease III (1 μg) or hmUDG (15 μg) in a final volume of 100 μl for 1 h at 37°C . The final reaction mixture contained 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, 0.1 mg/ml BSA. The reaction was stopped by addition of 20 μl BSA (500 μg) and 500 μl cold acetone. Acetone-insoluble material was separated by centrifugation and the acetone-soluble material was dried by evaporation under nitrogen, resuspended in 200 μl water and analyzed for the presence of ^3H -containing material by HPLC.

Enzymatic hydrolysis of DNA co-polymers to 2'-deoxyribonucleosides

DNA co-polymer in TKE buffer was made 0.015 M with respect to MgCl_2 , followed by treatment at 37°C with DNase I, alkaline phosphatase, spleen and snake venom phosphodiesterases and then alkaline phosphatase again overnight (14). The hydrolysates were precipitated with 5 vol acetone and centrifuged. Supernatants were evaporated and the residues dissolved in water and analyzed by HPLC.

HPLC analysis of ^3H -containing material released from co-polymers by endonuclease III or by enzymatic hydrolysis

Samples were analyzed on a 5 μm Ultrasphere ODS column using 50 mM ammonium formate, 0.5% methanol, pH 6.8, as the isocratic eluent at a rate of 1 ml/min. UV marker compounds thymine and 5-methylcytosine and radioactive marker compounds [^{14}C]thymine glycol and [^{14}C]thymidine glycol were added to the samples prior to HPLC analysis, as described in the text. Elution of radioactive material was monitored through the use of a Radiomatic Flo-One in-line radioactivity detector and elution of UV markers was monitored by on-line UV absorbance at 254 nm.

Oxidation of 5-methylcytosine with KMnO_4

Oxidation of 5-methylcytosine with KMnO_4 was carried out using the methodology previously described for oxidation of thymine to thymine glycol (14), except that the oxidation products were purified by repeated HPLC separations, instead of by an LH-20 column.

NMR spectroscopy

NMR spectral analysis of the products of KMnO_4 oxidation was performed using a Varian Unity 500 with d_6 -dimethylsulfoxide as solvent.

RESULTS

Synthesis of radiolabeled alternating DNA co-polymers

The formation of 5-[^3H]methylcytosine within the co-polymer poly(dG-[^3H]dmC) was confirmed by enzymatically digesting a sample of the co-polymer and measuring the yield of 5-[^3H]methylcytosine as the 2'-deoxyribonucleoside by HPLC analysis. After enzymatic hydrolysis the hydrolysates were precipitated with 5 vol acetone and centrifuged. Supernatants were collected and evaporated and the residues dissolved in water, mixed with

Figure 1. HPLC profile of acetone-soluble products released from γ -irradiated poly(dG-[^3H]dmC) by endonuclease III. The upper panel shows the ^3H radioactivity released by endonuclease III from poly(dG-[^3H]dmC). The middle panel shows the ^{14}C -labeled radioactive marker thymine glycol and the lower panel shows the A_{254} of UV marker 5-methylcytosine, both of which were added to the sample prior to HPLC analysis. The ordinate of the upper panels is d.p.m. The ordinate of the UV panel is an arbitrary sensitivity scale. The abscissa is time in minutes.

authentic 2'-deoxy-5-methylcytidine as a UV marker and analyzed by HPLC. Of the recovered radioactivity 99.6% co-eluted with authentic 2'-deoxy-5-methylcytidine (retention time 24.1 min). The specific activity of the co-polymer was typically $1.5\text{--}2 \times 10^6$ d.p.m./ μg , indicating that $\sim 40\%$ of cytosine residues in the co-polymer were methylated.

Characterization of the chemical identity of ^3H -containing material released from oxidized poly(dG-[^3H]dmC) by endonuclease III

Poly(dG-[^3H]dmC) was γ -irradiated, purified and then incubated with 1 μg endonuclease III. The acetone-soluble products of this reaction were then analyzed by HPLC. In this HPLC system thymine glycol and 5-methylcytosine eluted at 4.3 and 7.8 min respectively. A major peak co-eluting with authentic marker [^{14}C]thymine glycol was recovered (Fig. 1). Another minor peak which co-eluted with 5-methylcytosine was also recovered, but it was also present in the oxidized control sample incubated without endonuclease III, indicating that it represented post-irradiation non-enzymatically released material. After 400 Gy γ -irradiation ~ 10 pmol thymine glycol was released by endonuclease III from 1 μg co-polymer, indicating that $\sim 0.6\%$ of the total 5-methylcytosine residues in DNA were oxidized.

Oxidation of poly(dG-[^3H]dmC) with 2.8 mM H_2O_2 , 50 μM FeCl_3 and 200 μM ascorbate generated exactly the same results. The HPLC profile (Fig. 2) shows that the product released by endonuclease III from H_2O_2 -oxidized poly(dG-[^3H]dmC) co-eluted with authentic [^{14}C]thymine glycol. Oxidation of the co-polymer for 1 h resulted in the conversion of 1.5% of the total 5-methylcytosine residues to thymine glycol.

Characterization of the chemical identity of ^3H -containing material released from oxidized poly(dG-[^3H]dmC) by enzymatic hydrolysis

The enzymatic hydrolysis of DNA co-polymers was performed to determine if there was additional oxidative damage not

Figure 2. HPLC profile of acetone-soluble products released from H_2O_2 -oxidized poly(dG-[^3H]dmC) by endonuclease III. The upper panel shows the ^3H radioactivity released by endonuclease III from poly(dG-[^3H]dmC). The middle panel shows the ^{14}C -labeled radioactive marker thymine glycol and the lower panel shows the A_{254} of UV marker 5-methylcytosine, both of which were added to the sample prior to HPLC analysis. The ordinate of the upper panels is d.p.m. The ordinate of the UV panel is an arbitrary sensitivity scale. The abscissa is time in minutes.

released by endonuclease III formed in γ -irradiated poly(dG-[^3H]dmC). After enzymatic hydrolysis the HPLC analysis profile showed that, besides 5-methylcytosine itself, which contained $>95\%$ of the total radioactivity, three other major peaks were identified as thymine glycol, 5-hydroxymethylcytidine and thymine. There were also some other early eluting minor peaks whose identity is uncertain. The amount of the three modified pyrimidine bases released from non-irradiated and irradiated co-polymer are summarized in Table 1. The results indicate that thymine glycol was the main product of oxidation of 5-methylcytosine residues in DNA.

Table 1. The amount of modified bases in non-irradiated and γ -irradiated (400 Gy) Poly(dG-[^3H]dmC) detected by HPLC analysis after enzymatic hydrolysis

Modified Bases	Non-irradiated	γ -Irradiated
Thymine Glycol	0	1.5%
5-Hydroxymethylcytidine	1.0%	1.1%
Thymine	0.6%	0.8%

The data demonstrate a high rate of spontaneous hydrolytic deamination of 5-methylcytosine to thymine, because large amounts of thymine were present in digests of both non-irradiated and irradiated samples. The deamination rate for 5-methylcytidine to thymine in the alternating co-polymer was calculated to be $4 \times 10^{-8}/\text{s}$. The amount of thymine formed during enzymatic digestion of the irradiated sample did not increase significantly when compared with the non-irradiated sample, indicating that exposure to γ -irradiation did not lead to hydrolytic deamination of 5-methylcytosine without concomitant oxidation of the 5,6 double bond.

5-Hydroxymethylcytidine was also identified in the digest. This was formed from 5-[methyl- ^3H]methylcytosine via the transmutation of ^3H to ^3He by β -decay, leading to formation of the 5-hydroxymethyl group by the same mechanism as formation

Figure 3. Plot of the amount of thymine glycol released by endonuclease III versus γ -irradiation dose from poly(dA-[3 H]dT) ($\diamond$) and poly(dG-[3 H]dmc) ($\bullet$).

of 5-hydroxymethyluracil from [methyl- 3 H]thymine (17). The amount of 5-hydroxymethylcytidine in the irradiated sample did not increase significantly over that in the unirradiated sample, indicating that there was little net oxidation of the exocyclic 5-methyl group under the conditions employed.

Comparison of sensitivity to oxidation of poly(dG-[3 H]dmc) and poly(dA-[3 H]dT)

It has been well established that oxidation of poly(dA-[3 H]dT) results in the formation of thymine glycol from thymine (18). We compared the sensitivity to oxidation of 5-methylcytosine and thymine by comparing formation of thymine glycol in poly(dG-[3 H]dmc) to that formed in poly(dA-[3 H]dT) after increasing doses of γ -irradiation followed by incubation with endonuclease III. The amount of thymine glycol released from both co-polymers by endonuclease III increased with incident dose, as shown in Figure 3. The data indicate that 5-methylcytosine and thymine residues in DNA are equally susceptible to radiation-induced oxidation in DNA.

Chemical oxidation of 5-methylcytosine

The base 5-methylcytosine was oxidized with KMnO_4 in an attempt to synthesize 5-methylcytosine glycol to serve as a marker compound. After oxidation the products were separated by HPLC on a semi-preparative ODS column with 0.01% triethylamine eluting solution at a flow rate of 2 ml/min. The products were monitored by UV absorbance at 230 nm. For each HPLC run a total of 50×1 ml fractions were collected. The fractions corresponding to the first of four peaks, which was the major peak, were collected, pooled, concentrated and re-purified with HPLC. The NMR spectrum of this compound had signals at 1.12 (5- CH_3), 4.28 (6-H), 5.15 (5-OH), 6.04 (6-OH), 8.08 (1-NH) and 10.02 (3-NH). These values were identical to those obtained by us (14) for thymine glycol obtained by oxidation of thymine under conditions identical to those used here.

DISCUSSION

Thymine glycol was virtually the only product formed as a result of oxidation of 5-methylcytosine residues within a DNA alternating co-polymer. It has previously been shown that oxidation of cytosine in DNA results in formation of cytosine glycol. This oxidative derivative is very unstable, undergoing deamination to form uracil glycol, dehydration to form 5-hydroxycytosine or both dehydration and deamination to form 5-hydroxyuracil. *In vitro* γ -irradiation of calf thymus DNA yielded all of the above three products in approximately equimolar amounts (16). Thus in those experiments there was ~67% deamination of oxidized cytosine residues in DNA. The formation of uracil glycol from cytosine is analogous to formation of thymine glycol from 5-methylcytosine. 5-Hydroxy compounds are precluded from being formed from 5-methylcytosine because of the presence of the 5-methyl group.

We are not certain whether thymine glycol is formed from 5-methylcytosine via a concerted mechanism (oxidation and deamination), as shown in Figure 4 reaction (A), or via the formation of 5-methylcytosine glycol as an unstable intermediate, as in reaction (B). Oxidized 2'-deoxyribonucleoside-5-methylcytidine glycol has been synthesized by C. Bienvenu and J. Cadet (personal communication) via bromination followed by hydrolysis (15), but it proved unstable and underwent rapid deamination to thymidine glycol. The synthesis of an unstable 2'-deoxyribose-5-methylcytidine glycol suggests that the oxidation reaction we observed could occur via a two-step mechanism.

However, had 5-methylcytosine glycol been formed in the oxidized DNA co-polymers we would not have been able to detect it by nuclease digestion. By the time the overnight digestion at 37°C and HPLC analysis procedures were complete most, if not all, of the 2'-deoxyribose-5-methylcytidine glycol would have decomposed to thymidine glycol. The amount of residual 2'-deoxyribose-5-methylcytidine glycol would have been too small to be detected with certainty by our in-line scintillation counting system.

Was 5-methylcytosine glycol released by endonuclease III? Incubation of oxidized co-polymer with the repair enzyme was begun shortly after oxidation and was only for a few hours, in contrast to the overnight nuclease digestion, so it is possible that there was residual 5-methylcytosine glycol present within the co-polymer. The HPLC profiles of the material released by endonuclease III from oxidized poly(dA-dT) and oxidized poly(dG-dmC) are virtually identical, displaying one major peak which coincides with authentic thymine glycol and two smaller peaks. This pattern was previously observed by us (18) and others (19) after endonuclease III incubation of oxidized poly(dA-dT). The nature of these minor peaks is uncertain. We know, from previous experiments, that neither of them are *trans*-thymine glycol, since at 37°C equilibrium favors the *cis* form to almost 100%. Nor is either one of them 5-hydroxy-5-methylhydantoin, which has a different retention time under these chromatographic conditions (18). In any case, even if one were 5-methylcytosine glycol, it is apparent that the major product released by endonuclease III was recovered as thymine glycol.

The recovery of thymine glycol as the main product of incubation with endonuclease III indicates either that deamination took place rapidly on the DNA backbone or that free base 5-methylcytosine glycol is so unstable that after release by endonuclease III from the DNA backbone it is quickly deaminated

Figure 4. Reaction scheme for oxidation and deamination of 5-methylcytosine in DNA and repair of products by endonuclease III.

to thymine glycol. This is shown as reaction (C) in Figure 4. Our attempt to make 5-methylcytosine glycol by oxidation with KMnO_4 was unsuccessful, yielding virtually 100% thymine glycol. Therefore, until the synthesis of 5-methylcytosine glycol is accomplished, its stability remains unknown.

The failure to observe significant amounts of thymine or 5-methylcytosine residues oxidized at the 5-methyl group is consistent with earlier data from several laboratories, including our own (20,21). Although there is ample evidence for radiation-induced hydroxyl radical-mediated abstraction of hydrogen from the 5-methyl group on the pyrimidine ring, the chemical restitution reaction in which a hydrogen radical reacts with the methylene radical resulting in reformation of the methyl group is highly favored under dilute aqueous conditions (20,21).

Our current data also demonstrate that 5-methylcytosine and thymine within DNA are equally susceptible to oxidation of their 5,6 double bond. Oxidation of the thymine moiety should not be particularly mutagenic, since the lesion pairs with adenine during replication *in vitro* and its effects are thought to be largely cytotoxic (6). In contrast, oxidation of 5-methylcytosine may be of greater biological significance, because rapid deamination would lead to a GC→AT transition mutation if the thymine glycol residue were by-passed via the insertion of adenine, with concomitant loss of a methylated site.

In summary, we have demonstrated that thymine glycol is the main product of ionizing irradiation or chemically mediated oxidation of 5-methylcytosine in DNA. The high susceptibility to oxidation and the significant deamination rate of oxidized 5-methylcytosine may contribute to the high mutagenic rate observed at mCpG doublets in genomic DNA.

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