Glyoxal, a major product of DNA oxidation, induces mutations at G:C sites on a shuttle vector plasmid replicated in mammalian cells

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ABSTRACT

Glyoxal is a major product of DNA oxidation in which Fenton-type oxygen free radical-forming systems are involved. To determine the mutation spectrum of glyoxal in mammalian cells and to compare the spectrum with those observed in other experimental systems, we analyzed mutations in a bacterial suppressor tRNA gene (supF) in the shuttle vector plasmid pMY189. We treated pMY189 with glyoxal and immediately transfected it into simian COS-7 cells. The cytotoxicity and mutation frequency increased according to the dose of glyoxal. The majority of glyoxal-induced mutations (48%) were single-base substitutions. Among them, G:C→T:A transversions were predominant, followed by G:C→C:G transversions and G:C→A:T transitions. A:T→T:A transversions were also observed. Mutational hotspots within the supF gene were detected. These results suggest that glyoxal may play an important role in mutagenesis induced by oxygen free radicals.

INTRODUCTION

Oxygen free radicals are produced by a variety of normal metabolic processes, as well as by environmental mutagens, and have been hypothesized to contribute to the pathogenesis of many age-related human diseases, including cancer (1). In the mutagenic process, oxygen free radicals are thought to induce mutations through the formation of DNA damage that results in misincorporation during replication. The DNA damage caused by oxygen free radicals has been studied (2) and the mutagenicities of some lesions have been reported (3–7).

We previously showed that a mutagen, glyoxal (Fig. 1), was generated from DNA by exposure to a Fenton-type oxygen radical-forming system (FeSO₄–EDTA–O₂) (8) and that the yield of glyoxal was much higher (17-fold) than that of 8-hydroxydeoxyguanosine (8-OH-dG) (9). Moreover, the formation of glyoxal was estimated to be 13-fold more than that of 8-OH-dG when mixtures of deoxynucleosides were treated (Murata-Kamiya et al., unpublished results). It is possible that the glyoxal generated from DNA and DNA precursors immediately reacts with the DNA and the precursors to cause base modifications. Indeed, glyoxal reacts with a guanine base to form a tricyclic compound (glyoxal–dG, Fig. 1; 10,11). It was found that this modified nucleoside moiety is more prevalent than 8-OH-dG residues in DNA treated with oxygen radicals (Kasai et al., unpublished results). Thus, the formation of glyoxal from DNA and its precursors by oxygen free radicals may be involved in mutagenesis and carcinogenesis induced by oxygen radicals. Glyoxal is known to be mutagenic in Salmonella typhimurium strains TA100, TA102 and TA104 (9,12–14). Recently we found that glyoxal induces mutations at G:C base pairs, in a study using a set of seven S.typhimurium strains (TA7001–TA7006 and TA98; 15). Moreover, glyoxal induces mutations mainly at G:C base pairs in wild-type Escherichia coli (16). To obtain more knowledge about glyoxal-induced mutagenesis and to clarify the actual role of glyoxal in oxygen radical-induced mutagenesis it is important to analyze the spectra of mutations induced by glyoxal in mammalian cells.

The shuttle vector plasmid pMY189 (17) is derived from pZ189 (18), which has been widely used in assessing mutations in mammalian and E.coli cells (17,19–23). pMY189 carries a bacterial suppressor tRNA gene, supF, as a target gene (176 bp). More than half of the bases in the tRNA molecule are involved in the base pairing that maintains the tRNA secondary structure and many of the unpaired bases in the loops are involved in specific interactions with the tRNA synthetase or ribosomes or in codon recognition (24,25). In fact, single-base substitutions that inactivate gene function were identified in almost all of the positions corresponding to the tRNA molecule (17). Moreover, the entire DNA sequence of the gene can be determined in one operation. We transfected glyoxal-treated pMY189 DNA into simian kidney (COS-7) cells and mutations within the supF gene were detected with an indicator bacterial system (21). We show that glyoxal induced predominantly G:C→T:A transversions, followed by G:C→C:G, A:T→T:A and G:C→A:T mutations.

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MATERIALS AND METHODS

Cells, bacterial strains and plasmids
Simian kidney (COS-7) cells were obtained from the RIKEN cell bank (Tsukuba, Japan). Indicator E. coli KS40 cells [gyrA, lacZ(am), CA7070, hsdR, hsdM, Δ(araABC-leu)7679, galU, galK, rpsL, thi] containing pKY241 (KS40/pKY241) and the shuttle vector plasmid pMY189 were kind gifts from Dr T. Matsuda. Escherichia coli HB101 cells [supE44, hsdR2 (r− m−), recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1, leuB6, thi-1] were purchased from Nippon Gene Co.

Media
All chemicals not specifically mentioned were purchased from Wako Pure Chemical Industries Co. Dulbecco’s modified Eagle medium (DMEM) and fetal calf serum were obtained from Sigma (St. Louis, MO, USA). Luria-Bertani (LB) medium and LB plates were prepared as described (26). Chemicals listed below were used for LB medium and LB plates as indicated: 50 μg/ml nalidixic acid; 30 μg/ml chloramphenicol; 150 μg/ml ampicillin (Sigma); 1 μM isopropyl-β-D-thiogalactopyranoside (IPTG, Nacalai Tesque Inc.); 0.008% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

Treatment of plasmid with glyoxal
Plasmid pMY189 (400 ng) was treated with 0–150 μg/ml glyoxal (Nacalai Tesque Inc.) in a total volume of 100 μl at 37 °C for 60 min (pH 6.4). The unreacted glyoxal was removed by gel filtration using a G25-SP column (Pharmacia Biotech Inc.). Glyoxal-treated pMY189 was immediately introduced into COS-7 cells as described below.

DNA transfection into COS-7 cells and recovery of plasmid
COS-7 cells (5 × 10^5 cells) were plated into a 6 cm dish and were cultured in DMEM medium supplemented with 10% fetal calf serum at 37 °C under a 5% CO₂ atmosphere. After an incubation for 24 h, the glyoxal-treated vectors (10 ng) were transfected into the cultured COS-7 cells using Lipofectamine™ (Life Technologies Inc.) according to the supplier’s recommendations. After 24–48 h, plasmid amplified in the cells was recovered by the method of Stary and Sarasin (27). The recovered DNA was digested with DpnI (New England Biolabs Inc.) to eliminate the unreplicated plasmids, which retained the bacterial methylation pattern. After removal of proteins by passage through an Ultrafree-Probind MC filter unit (Millipore Co.), the DNA was purified by ethanol precipitation.

Determination of cytotoxicity
The plasmid DNA recovered from COS-7 cells was introduced into E. coli strain HB101 by the CaCl₂ method. Portions of the culture of transformed E. coli were plated onto a LB plate containing ampicillin and were incubated at 37 °C overnight.

Selection of the mutated supF gene
The plasmid DNA recovered from COS-7 cells was introduced into the indicator bacteria KS40/pKY241 (21) by the CaCl₂ method. To select E. coli with a mutated supF gene, the transformed cells were plated onto LB plates containing nalidixic acid, ampicillin, chloramphenicol, IPTG and X-gal and were incubated at 37 °C overnight. A white colony on these plates indicated a supF mutant. To determine the total number of transformants, a portion of the transformed cells was plated on a LB plate containing ampicillin and chloramphenicol. After a 24 h incubation at 37 °C, the colonies were counted and the mutation frequencies were calculated.

The mutant E. coli colonies were inoculated into 0.5 ml LB medium containing ampicillin and the cultures incubated at 37 °C overnight. Plasmid DNAs were extracted from the cells by the alkaline lysis method.

DNA sequencing
Upper (5’-AGTGCCACCTGACATCT-3’, 5419–5436 of pMY189) and lower (5’-CAGCGATTACGCCAGA-3’, 278–261) primers were purchased from Hokkaido System Science Co. (Sapporo, Japan) in purified form. Sequencing reactions were carried out using an Applied Biosystems Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Co.) on a thermal cycler (Temp-Tronic; Thermolyne) by the recommended protocol. An Applied Biosystems model 373S DNA Sequencer and 6.75% LongRange™ gels (FMC BioProducts) were used for the analysis.

RESULTS

Glyoxal is mutagenic in mammalian cells
The shuttle vector plasmid pMY189 was treated with various doses of glyoxal and both the cytotoxicity and mutation frequency (MF) in simian COS-7 cells were determined. The plasmid DNA recovered from COS-7 cells was transfected into E. coli HB101. Since the number of E. coli colonies is proportional to the amount of DNA replicated in COS-7 cells under our conditions (data not shown), the relative transforming efficiency is an indicator of cytotoxicity. The number of E. coli colonies significantly decreased in a glyoxal dose-dependent manner (Fig. 2A), showing that replication was (partially) blocked by glyoxal-adducted residues. On the other hand, the MF (defined by...
the supF mutant fraction divided by the total transformant fraction) increased with glyoxal treatment (Fig. 2B). At a dose of 100 µg glyoxal, the replicated plasmid was only 2.7% of the control and the MF reached 3.8 × 10^{-3} after treatment. This MF value was 4.6-fold above background (8.2 × 10^{-4}). At the higher doses of glyoxal, the relative transforming efficiency was further decreased and the MF was increased. Thus it is clear that glyoxal induced mutations in COS-7 cells. We chose 50–150 µg as the dose for selection of mutants and characterization of the mutations, based on both the MF and the number of colonies obtained. We selected 9, 28, 59 and 2 colonies from 50, 100, 120 and 150 µg experiments respectively and the average MF was 9.1 × 10^{-3} (11-fold of the control).

Mutations induced by glyoxal at G:C sites

Forty eight independent spontaneous mutants and 98 glyoxal-induced supF mutants were selected and the types of mutations were characterized by sequence analysis. The results are summarized in Table 1.

In the mutants induced by glyoxal, single-base substitutions were predominant (48%), followed by multi-base deletions (32%, average deleted sequence 133 bp, range, 29–233 bp). Other mutations included multiple mutations at G:C sites (11%), tandem mutations at GG sequences (4%) and G:C base pair deletions (4%). Among the single-base substitutions, 83% of the mutations occurred at G:C base pairs and G:C→T:A transversions were predominant (48% of the single-base substitutions), followed by G:C→C:G transversions (21%). A G:C→A:T transition was detected in only one case.

The overall spectra of both the spontaneous and the glyoxal-induced mutations were similar. However, the average MF of the glyoxal-induced mutations used for the sequence analysis was 11-fold higher than the spontaneous mutation frequency. Thus, almost all of the induced mutations detected are probably derived from reactions of DNA and glyoxal.

The distribution of the single-base substitutions detected in the supF gene is shown in Figure 3. In both the spontaneous and the glyoxal-induced mutation spectra, the distributions of the mutations were not random and some mutational hotspots were observed (Fig. 3). In the spontaneous mutations, the majority (48%) of the single-base substitutions occurred at position 160 of pMY189, where the G:C→T:A transversions occurred. Three other, minor hotspots (positions 123, 133 and 139) were also observed. In the glyoxal-induced mutations, two major hotspots (positions 133 and 159) and two minor hotspots (positions 162 and 168) were observed. The clearest hotspot was located at position 133 (35% of the single-base substitutions), where G:C→T:A transversions occurred, and another hotspot was located at position 159 (27%), where G:C→C:G mutations occurred. Although the overall spectra of the spontaneous and glyoxal-induced mutations were similar, the distributions were very different. Therefore, the mutations obtained should be derived from modification(s) by glyoxal.

The distributions of the tandem mutations and the multiple mutations induced by glyoxal are shown in Figure 4. The majority (47%) of the mutations occurred at positions 99–103. This shows that the mutational hotspots were different among the various types of mutations. In the spontaneous mutations, the hotspots were different between the single-base mutations and the tandem and multiple mutations (see legend to Table 1). For the multiple mutations induced by glyoxal, all of the mutations occurred at G:C sites. Moreover, the mutated G or C residues in a single gene

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Cases found (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-base substitution</td>
<td>Glyoxal-induced</td>
</tr>
<tr>
<td>G:C→T:A</td>
<td>23 (23)</td>
</tr>
<tr>
<td>G:C→G</td>
<td>11 (11)</td>
</tr>
<tr>
<td>G:C→A:T</td>
<td>6 (6)</td>
</tr>
<tr>
<td>A:T→T:A</td>
<td>8 (8)</td>
</tr>
</tbody>
</table>

Tandem mutation

| G:C→T:T(GG) | 4 (4) | 2 (2) |
| G:C→T:T(GG) | 2 (2) | 1 (1) |
| G:C→T:T(GG) | 1 (1) | 0 (0) |
| G:C→T:T(GG) | 0 (0) | 1 (2) |

| A:T→T:A | 4 (4) | 4 (4) |
| Multi-base deletion | 31 (31) | 12 (12) |
| Multiple mutation | 11 (11) | 3 (3) |
| Total | 98 | 48 |

*All of the mutations occurred at G:C sites. In glyoxal-induced mutants, 12 G:C→A:T mutations, nine G:C→T:A mutations and three G:C→C:G mutations were observed (position 5, see Fig. 4). In spontaneous mutants, G:C→A:T and G:C→C:G (positions 172 and 174) were observed within two colonies and a one base deletion at a G:C site and G:C→A:T (positions 102–105 and 113) were observed within one colony.

Table 1. Types of mutations in the supF gene of shuttle vector plasmid pMY189 replicated in COS-7 cells

Figure 2. Glyoxal-induced (A) cell death and (B) mutation frequency in COS-7 cells. (A) Plasmids replicated in COS-7 cells were transfected into E.coli HB101 as in Materials and Methods. The number of transformants was proportional to the amount of plasmid DNA recovered. (B) The mutation frequencies in the supF gene at the same time point were calculated according to Materials and Methods. Experiments were done at least in triplicate and the mean values are represented.
Figure 3. The overall distribution of single-base mutations, which includes single-base substitutions and G:C deletions, detected in the supF gene. Spontaneous and the glyoxal-induced mutations are shown above and below the sequence respectively. The base pair substitutions are indicated as single letters. The G:C deletions are represented by open triangles. When a deletion occurs in a group of identical bases, the relevant nucleotides are underlined. When specific mutations were found more than once, occurrence of the mutations is represented by the number. The anticodon (CTA) is indicated in bold.

Figure 4. The overall distribution of glyoxal-induced tandem and multiple mutations detected in the supF gene. Tandem mutations are shown above the sequence and multiple mutations are shown below the sequence. The anticodon (CTA) is indicated in bold.

were located in the same strand, i.e. the mutations were induced in the sequence of 5'-G----G-3' (Fig. 4). G:C→A:T transversions (12 of the 24 mutations) occurred more than G:C→T:A transversions (nine mutations) (Fig. 4). This fact is in contrast to the finding that G:C→T:A transversions were found more frequently than G:C→A:T mutations in the case of the single substituted mutants (Table 1). When compared with the 100 and 150 µg experiments, the frequencies of the tandem and multiple mutations increased with increasing glyoxal dose (data not shown). Thus, the tandem and multiple mutations appeared to be induced by glyoxal.

DISCUSSION

Glyoxal is a major product generated from DNA and its related compounds by Fenton-type oxygen free radical-forming systems (8). Glyoxal is also known to be produced in lipid peroxidation systems (28). In addition, glyoxal is present ubiquitously in beverages and foods, such as coffee, soy sauce and bean paste (29), and is found in cigarette smoke (30). Thus, glyoxal may be involved in many pathways of human carcinogenesis.

The predominant mutation induced by glyoxal was G:C→T:A transversions, followed by G:C→C:G and G:C→A:T mutations (Table 1). In various mutagenesis studies, among the 93 target sites reported previously, 54 sites are present in G:C base pairs and 39 sites are in A:T base pairs (17). The frequency of single-base substitutions detected at G:C sites was 5-fold higher than that at A:T sites in the present results. Thus, the induced mutations appeared to occur preferentially at G:C sites. This spectrum is consistent with our previous findings that glyoxal induced mutations at G:C sites in S.typhimurium (15). However, in the lacI gene of E.coli, glyoxal predominantly induced G:C→A:T and G:C→T:A mutations, and G:C→C:G transversions were rarely observed (16). On the other hand, G:C→A:T transitions were less frequent than G:C→C:G transversions in COS-7 cells (Table 1). Thus, the glyoxal-induced mutations found in mammalian cells are different from those in E.coli. Many DNA lesions, such as abasic sites (31–33) and propanoguanine (34), show different mutation spectra between E.coli and mammalian cells. This may be due to: (i) differences of the DNA polymerases in DNA replication; (ii) differences in the DNA repair pathways between E.coli and mammalian cells. On the other hand, A:T→T:A transversions, which were elicited in COS-7 cells (Table 1), were observed in E.coli at low frequency (16) and were not found in S.typhimurium (15). Therefore, the mutation spectra of a compound or a DNA adduct should be studied in different organisms.

The G:C→T:A transversion is a single-base substitution found frequently in DNA treated with oxygen radicals (20,22,23,35–37). This mutation is of the same type as 8-OH-dG, which can...
pair with dA during DNA synthesis, resulting in G:C→T:A transversions (4,5). Although it has been suggested that the 8-OH-dG formed in DNA by oxygen radicals is a main factor in G:C→T:A transversions, our data suggest that G:C→T:A mutations may be due to glyoxal, at least in part. Indeed, Akasaka and Yamamoto reported that G:C→T:A transversions occur by a lipid peroxidation system, without an increase in the formation of 8-OH-dG in DNA (38). It is possible that glyoxal is involved in this mutagenesis pathway. Since glyoxal is formed more readily than 8-OH-dG (9; Murata-Kamiya et al., unpublished results), glyoxal may be a main factor in G:C→T:A transversions. G:C→C:G transversion is the second most frequent class of single-base substitution induced by glyoxal. Formation of this type of mutation has been observed in mutagenesis experiments with Fe²⁺, hydrogen peroxide, methylene blue and lipid peroxidation systems (20,22,23,35–38) and the actual origin of this mutation has not been identified. It is possible that glyoxal also plays an important role in the generation of G:C→C:G transversions induced by oxygen radicals, at least in mammalian cells. Therefore, glyoxal may be one of the origins of both G:C→T:A and G:C→C:G transversions induced by oxygen radicals (20,22, 23,35–38).

Glyoxal induced multiple mutations at G:C sites (Fig. 4). Twelve of the 24 mutations found were G:C→A:T transitions and G:C→T:A transversions occurred in nine mutations. This is in contrast to the observation that G:C→T:A mutations were 4-fold more prevalent than G:C→A:T mutations in the single-base mutants (Table 1). This fact may indicate that the types of glyoxal modifications that elicited single-base and multiple substitutions are different.

Glyoxal reacts with a guanine base to form a tricyclic glyoxal–dG adduct (Fig. 1; 10,11). When the adduct adopts the syn conformation, it can pair with protonated adenine (15). It is possible that the observed G:C→T:A mutations were due to glyoxal–dG (syn):dA* pair formation. This type of hydrogen bonding has been elucidated by NMR studies of the base pair between propanodeoxyguanosine, which has a structure similar to glyoxal–dG, and protonated dA (39). The glyoxal–dG adduct in the syn conformation can also base pair with guanine and induce G:C→C:G mutations (15).

Mutations in the supF gene induced by various mutagens have been reported. No report perfectly agrees with our data, however, some hotspots were the same. Position 160, a major hotspot for spontaneous mutations in this study, is also one of the hotspots of G:C→T:A transversions in H₂O₂-induced mutations in E.coli (22). Position 133, the first position of the anticodon, is one of the major hotspots of glyoxal-induced mutations and the induction of mutations at this position has been previously reported (22,23,38,40). Position 159, one of the major hotspots in the induced mutation spectrum in this study, was also a hotspot for 2-chloroacetalddehyde-induced mutations in human cells (17).

The nearest neighboring bases of the putative glyoxal-modified dG residues (Fig. 3) were very different from those analyzed in E.coli (16). In both E.coli and COS-7 cells, some sequence selectivity was observed for the single-base substitutions that occurred at G:C sites. In COS-7 cells, the 5′-site of the mutated base G was predominantly A (5′-AG, mutated base underlined). In E.coli, however, 5′-TG (G:C→A:T mutations) or 5′-CG and 5′-GG (G:C→T:A mutations) were predominant. On the other hand, a G:C-3′ sequence was found most frequently in all of the mutants with G:C→A:T and G:C→T:A mutations. The 3′-site of the mutated base G varied according to the kind of mutation in COS-7 cells. In the case of G:C→T:A transversions, a GA-3′ sequence was predominant. In both G:C→C:G and G:C→A:T mutations, a GG-3′ sequence was predominant. Although the reasons for these differences are unknown, it is possible that glyoxal produced other DNA lesions, with different sequence selectivities, in addition to the tricyclic dG adduct and that the mutation spectra of these lesions differed in COS-7 and E.coli cells.

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REFERENCES