DNA-Protein Cross-link Formation Mediated by Oxanine

A NOVEL GENOTOXIC MECHANISM OF NITRIC OXIDE-INDUCED DNA DAMAGE

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Chronic inflammation is a risk factor for many human cancers, and nitric oxide (NO) produced in inflamed tissues has been proposed to cause DNA damage via nitrosation or oxidation of base moieties. Thus, NO-induced DNA damage could be relevant to carcinogenesis associated with chronic inflammation. In this report, we report a novel genotoxic mechanism of NO that involves DNA-protein cross-links (DPCs) induced by oxanine (Oxa), a major NO-induced guanine lesion. When a duplex DNA containing Oxa at the site-specific position was incubated with DNA-binding proteins such as histone, high mobility group (HMG) protein, and DNA glycosylases, DPCs were formed between Oxa and protein. The rate of DPC formation with DNA glycosylases was approximately two orders of magnitude higher than that with histone and HMG protein. Analysis of the reactivity of individual amino acids to Oxa suggested that DPC formation occurred between Oxa and side chains of lysine or arginine in the protein. A HeLa cell extract also gave rise to two major DPCs when incubated with DNA-containing Oxa. These results reveal a dual aspect of Oxa as causal damage of DPC formation and as a suicide substrate of DNA repair enzymes, both of which could pose a threat to the genetic and structural integrity of DNA, hence potentially leading to carcinogenesis.

Nitric oxide (NO) synthesized from l-arginine by three isoforms of NO synthase mediates a variety of regulatory functions in vivo (1, 2). A high flux of NO is produced by macrophages expressing inducible NO synthase during inflammatory responses (3). Because chronic inflammation has been long recognized as a risk factor for many human cancers, it has been postulated that NO is carcinogenic by virtue of its ability to induce DNA damage (4–7). Although NO itself is not reactive to DNA, this molecule is converted to reactive nitrogen oxide species such as peroxynitrite (ONOO−) and nitrous anhydride (N2O3). ONOO− is a powerful oxidizing agent formed by the reaction with the superoxide anion (O2−) that is simultaneously excreted from macrophages. ONOO− primarily oxidizes guanine to 7,8-dihydro-8-oxoguanine (8oxoG) (8), further degradation products of 8oxoG (9), and 8-nitroguanine (10, 11). 8oxoG is a potent mutagenic lesion inducing GC → TA transversions, whereas 8-nitroguanine readily undergoes depurination to yield an abasic site, a potent lethal as well as mutagenic lesion. N2O3 is a powerful nitrosating agent that is formed by autoxidation of NO in the presence of oxygen. Nitrosation of the primary amino group of guanine, adenosine, and cytosine by N2O3 induces deamination, resulting in xanthine (Xan), hypoxanthine (Hx), and uracil (U), respectively (12, 13). Nitrous acid (HNO2) also induces deamination of guanine, adenosine, and cytosine by a similar mechanism (14).

We have previously identified a novel reaction product, oxanine (Oxa), that is formed by the nitrosation of guanine with NO or nitrous acid (15, 16). Oxa and Xan were formed at a molar ratio of 1:3 when 2’-deoxyguanosine or DNA was treated with NO or nitrous acid. Oxa is also formed by incubation with N-nitrosoindoles (17, 18), showing that N-nitroso compounds produced by nitrosation of secondary amines by NO or nitrous acid can mediate Oxa formation. Although Oxa can be mutagenic and cytotoxic by directly affecting DNA replication or the stability of duplex DNA (19, 20), there is another possibility: the O-acetylurea structure of Oxa is fairly reactive and can form an adduct or a DNA-protein cross-link (DPC) with nucleophilic cellular molecules (Fig. 1A). Indeed, we have recently shown in a model reaction that an Oxa-glycine adduct was formed when 2’-deoxyoxanosine and concentrated glycine were incubated in aqueous solution (21). Similarly, a diazoate derivative of 2’-deoxyctydine, a reaction intermediate formed by NO or nitrous acid (22), also reacts with lysine and its homopolymer to yield covalent adducts (23) (Fig. 1B). Interestingly, the results from several laboratories using repair-deficient Escherichia coli strains suggest that the principal DNA repair pathway for counteracting the mutagenic or cytotoxic effect of NO and nitrous acid is not base excision repair but nucleotide excision or recombination repair (14, 24–28). Considering the bulky nature of DPCs and the covalent adducts potentially formed by Oxa and cytosine diazoate, it is likely that these lesions are processed by nucleotide excision or recombination repair in cells (29, 30). Consistent with this notion, it has been shown recently that UvrABC nuclease incises DPC containing a covalent T4 endonuclease adduct with a moderate efficiency (31). Thus, DPCs and covalent adducts induced by NO or nitrous acid may exert mutagenic or cytotoxic effects in the...
absence of appropriate repair mechanisms (14, 24–28). However, except for the model reactions described above (21, 22), no studies have been performed to clarify whether DPC or adducts are formed between Oxa or cytosine diazoate in DNA and cellular components.

In the present study, we prepared a duplex oligonucleotide containing site-specific Oxa and examined the reactivity to proteins and polyamines. We report here that Oxa in DNA reacts with nuclear proteins such as histone, high mobility group (HMG) protein, and certain types of DNA repair enzymes (i.e. DNA glycosylases) as well as polyamines to form DPC. DPC formation is also observed in the incubation with the HeLa cell extract. The efficiencies of DPC formation with histone and DNA glycosylases differ dramatically, suggesting two distinct mechanisms of DPC formation.

EXPERIMENTAL PROCEDURES

Chemicals and Enzymes—2′-Deoxynoxanosine 5′-triphosphate (dOTP) was synthesized and purified as reported previously (19). Oligonucleotides with normal constituents were synthesized by the standard phosphoramidite method. A 25-mer oligonucleotide (25X) containing 8oxoG was synthesized and purified as reported previously (19). Oligonucleophosphorimaging analyzer. The reactions with DNA repair enzymes were performed in a similar manner. The enzymes used were DNA glycosylase (final concentration, 2.7 M each of all proteins (final concentration, 2.5 M) in an appropriate buffer (30 °C for up to 1 h). The composition of the incubation buffer was 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, and 1 mM DTDA for Endo III, Endo VIII, and Fpg; and 70 mM Hepes-KOH (pH 7.8), 1 mM EDTA, and 5 mM mercaptoethanol for AlkA. Products were analyzed by SDS-PAGE as described for histone. When the reaction was performed with inactivated enzyme (or histone), proteins (hOGG1, AlkA, Endo VIII, and histone mixture) were heat-denatured at 80 °C for 10 min prior to incubation. The heat treatment of hOGG1, AlkA, and histone did not result in significant changes in protein concentrations as judged from SDS-PAGE analysis. However, the concentration of Endo VIII was decreased to one-third of that before heat treatment probably due to aggregation of the protein. Thus, the concentration of Endo VIII was increased (3-fold) in the reaction with hOGG1, AlkA, Endo VIII, and histone mixture)

Preparation of an Oligonucleotide Containing Oxa—Oxa was site-specifically incorporated into an oligonucleotide by a DNA polymerase reaction using dOTP. A 15-mer primer (5′-CATCTGATCATCTCTCTC-3′) was 5′-labeled with 32P and annealed to a 25-mer template (3′-GATGC-TATCTGATGCCTGAGGAGACGG-5′; the underlined position template indicates where dOTP was incorporated). The template/primer (25 pmol; final concentration 62.5 nM) was incubated with dOTP (20 μM) and Pol I Kf (exo −) (50 units) in 66 mM Tris-HCl (pH 7.5, 1.5 mM 2-mercaptoethanol, and 6.6 mM MgCl2 (total volume, 400 μl) at 25 °C for 5 min (first-stage reaction). Subsequently, dCTP and dTTP (final concentration, 20 μM) were added to the reaction mixture and incubation was further continued at 25 °C for 40 min (second-stage reaction). DNA was purified by phenol extraction, ethanol precipitation, and gel filtration on a Sephadex G75 column (3 ml). Under these conditions, a single residue of Oxa was incorporated immediately after the original primer terminus (i.e. opposite the underlined “C” in the template). This was confirmed by PAGE analysis of the first-stage reaction product in a separate experiment. The resulting primer terminus containing Oxa was extended to a fully replicated product by incorporation of dCTP and dTTP. dOTP is not incorporated during the second-stage reaction, because Pol I Kf (exo −) inserts dOTP opposite template pyrimidines (C and T) but not purines (A and G) (19).

Reactions of 25O/C with Proteins—25O/C (final concentration, 4 nM) was incubated with 4 μg of a histone component (H1, H2A, H2B, H3, or H4; final concentrations, 6–12 μg), a mixture of 4 μg each of all components (H1–H4; final concentrations, 6–12 μg), or 2 μg of HMG protein (final concentration, 2.7 μg) in 10 mM phosphate buffer (pH 7.4, 30 μl) at 37 °C for up to 48 h. The sample was mixed with an equal volume of SDS-loading buffer (100 mM Tris-HCl (pH 6.8), 8% SDS, 24% glycerol, 0.02% SERVA BLUE G, and 4% 2-mercaptoethanol), heated, and separated by 10% SDS-PAGE. Molecular weight markers were also electrophoresed side by side: phosphorylase (Mw = 97,400), albumin (66,287), aldolase (42,400), carboxy anhydrase (30,000), trypsin inhibitor (20,100), and lysozyme (14,400). The gel was briefly stained with Coomassie Brilliant Blue (CBB), dried, and subjected to autoradiography. The radioactivity of the gel was measured using a Fuji BAS2000 phosphorimaging analyzer. The reactions with DNA repair enzymes were performed in a similar manner. The enzymes used were DNA glycosylases from human (hNTH1 and hOGG1), mouse (mMPG), and E. coli (Endo III, Endo VIII, Fpg, and AlkA). 25O/C (final concentration, 4 nM) was incubated with 2 μg of a DNA glycosylase (final concentrations, 1.7–2.2 μM) in an appropriate buffer (30 μl) at 37 °C for up to 1 h. The composition of the incubation buffer was 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, and 1 mM DTDA for hNTH1 and hOGG1; 50 mM Hepes-KOH (pH 7.8), 1 mM EDTA, 5 mM mercaptoethanol, and 10 mM KCl for mMPG; 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 1 mM EDTA for Endo III, Endo VIII, and Fpg; and 70 mM Hepes-KOH (pH 7.8), 1 mM EDTA, and 5 mM mercaptoethanol for AlkA. Products were analyzed by SDS-PAGE as described for histone. When the reaction was performed with inactivated enzyme (or histone), proteins (hOGG1, AlkA, Endo VIII, and histone mixture) were heat-denatured at 80 °C for 10 min prior to incubation. The heat treatment of hOGG1, AlkA, and histone did not result in significant changes in protein concentrations as judged from SDS-PAGE analysis. However, the concentration of Endo VIII was decreased to one-third of that before heat treatment probably due to aggregation of the protein. Thus, the concentration of Endo VIII was increased (3-fold) in the reaction with inactivated Endo VIII (see also “Results” for Fpg).

Protease and DNase Treatment of DPCs—25O/C and protein (histone H2B, Fpg, or Endo VIII) were incubated as described above. After incubation, an aliquot (2.5 μl) of the reaction mixture was further treated with trypsin, proteinase K, thrombin, DNase I, or Exo I in a buffer recommended by the suppliers (final volume, 10 μl) at 37 °C for 10 min to 1 h. Further details of the experiments are described in the figure. Products were analyzed by SDS-PAGE.

DNA-Protein Cross-links Induced by Oxanine

FIG. 1. Reaction schemes for adduct or cross-link formation of oxanine and cytosine diazoate. A, formation of oxanine by nitration of guanine and adduct or cross-link formation by the reaction with a cellular nucleophilic molecule. B, formation of cytosine diazoate by nitration of cytosine and adduct or cross-link formation by the reaction with a cellular nucleophilic molecule.
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**RESULTS**

**DPC Formation with Histone and HMG Protein—**25O/C was incubated with individual histone components (molar ratio of 25O/C:histone = 1:1,000–3,000) for 12–36 h, and the products were analyzed by SDS-PAGE. Fig. 2A shows the SDS-PAGE data for the sample after 36 h of incubation. All histone components (H1, H2A, H2B, H3, and H4) reacted with 25O/C to form DPCs so that new bands other than free DNA were observed in autoradiogram (Fig. 2A, right panel). The gel mobility of DPCs was lower than that of the corresponding free proteins as well as free DNA (Fig. 2A). The apparent $M_r$ values of free histones and their DPCs were determined from the gel mobility of free histones (CBB-stained bands) and that of DPCs (autoradiogram) (Fig. 3A). The differences in the $M_r$ between free histone components and the corresponding DPCs were 7,900–11,000, which corresponded to the approximate size of single-stranded 25O ($M_r = 8,100$). Thus, DPC comprised a histone component and 25O/C at a ratio of 1:1 before denaturation of the complex. DPC was not formed when the reaction was performed with 25G/C containing G in place of Oxa at the same site (data not shown), indicating that the cross-link reaction was specific for Oxa. Because the preparations of histones H2B and H3 contained histones H4 and H1/H2A as contaminants, respectively (Fig. 2A, left panel, marked bands), DPCs due to the contaminants also appeared as additional bands in the autoradiogram (Fig. 2A, right panel, marked bands). The apparent rate constant of cross-link formation of Oxa ($k_{app}$) was calculated from the hourly yield of DPC ([Cross-link product]/([25O/C] × [Protein (amino)]) in Table I) by applying second order reaction kinetics, where $k_{app} = [\text{Cross-link product}]/([25O/C] \times [\text{Protein (amino)})]$. The variation in $k_{app}$ for the histone components was at most 3-fold (lowest for H1 and highest for H4, Table I). 25O/C was also incubated with a mixture of all histone components under similar conditions. Each component formed DPC (Fig. 2B), but

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**Fig. 2. Cross-link formation between Oxa and histone or HMG protein.** A, 25O/C containing Oxa (25O was $\gamma$-labeled with $^{32}$P) was incubated with the indicated histone components (H1, H2A, H2B, H3, and H4) at 37°C for 36 h. Products were separated by SDS-PAGE. Left panel, SDS-gel stained with CBB. The band positions of the $M_r$ markers are indicated on the left. The bands marked with dots correspond to contaminated proteins (see text for details). Note that histone components (and HMG protein in C) did not exhibit the gel mobilities expected from their $M_r$ values due to the high basicity. Right panel, autoradiogram of the SDS-gel. The bands of free DNA and cross-linked products are indicated. The bands marked with dots were cross-linked products arising from contaminated protein. B, 25O/C was similarly incubated with a mixture of histone components (H1–H4) at 37°C for up to 36 h. Products were separated by SDS-PAGE. Left panel, CBB-stained SDS-gel for the sample with an incubation time of 36 h. The bands of histone components (H1–H4) are indicated. Right panel, autoradiogram of the SDS-gel for the samples with an incubation time of 0, 12, 24, or 36 h. C, same as B except that HMG protein was used instead of a histone mixture.

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**Reactions with HeLa Cell Extracts—**The nuclear cell extract was prepared from confluent HeLa cells. All procedures were performed at 4°C or on ice. The harvested cells were suspended in 5 volumes of 10 mM Hepes-KOH (pH 7.9), 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT. The extract was stored at 37°C for 20 min, and the supernatant was discarded. The precipitate containing cell nuclei was mixed with a half volume of low salt buffer (20 mM Hepes-KOH (pH 7.9), 1.5 mM MgCl₂, 1.2 M KCl, 0.2 mM PMSF, and 0.5 mM DTT). The cells were disrupted with a Dounce homogenizer (20 strokes). The sample was centrifuged (3,300 × g for 15 min), and the supernatant was discarded. The precipitate containing cell nuclei was mixed with a half volume of hypotonic buffer (20 mM Hepes-KOH (pH 7.9), 1.5 mM MgCl₂, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, and 25% glycerol) and kept standing for 30 min. The sample was centrifuged at 25,000 × g for 30 min, and the supernatant was dialyzed (M₆ cut-off = 3,000) against 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT for 5 h. The extract was stored as aliquots at −80°C. The protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL). 25O/C (final concentration, 1 mM) (Sigma, St. Louis, MO) in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT was added to the sample. The reaction was incubated with the indicated histone components (H1–H4) at 37°C for up to 48 h. After incubation, DNA was recovered by ethanol precipitation. The sample was mixed with formamide gel loading buffer, heated, and separated by 16% denaturing PAGE. The radioactivity of the gel was analyzed as described above. The reactions with monoamines (propylamine and dipropylamine) and polyamines (spermidine, spermine, and putrescine) were performed in a similar manner.
DNA-Protein Cross-links Induced by Oxanine

The total amount of DPC formed with the histone mixture was significantly lower (by about 5-fold) than the simple summation of DPC formed from individual reactions (e.g., compare Fig. 2A, right panel (all 36 h reactions), with Fig. 2B, right panel, at the 36 h reaction). Histones H2A and H2B form a stable dimer (H2A/H2B), and H3 and H4 form a stable tetramer ([H3/H4]) in the absence of DNA (40, 41). It is possible that the association between histone components might have masked reactive sites in the proteins or reduced the effective concentration of histones available for the reaction. HMG protein also reacted with 250/C to form DPC (Fig. 2C). The reaction conditions of HMG protein were similar to those for histone (molar ratio of 250/C:HMG = 1:675 and incubation time = 12–36 h). The DPC contained HMG protein and 250/C at a ratio of 1:1 as judged from the increase in the apparent M_r of DPC (Fig. 3A). The value of k_app with HMG protein was comparable to those of histone components (Table I).

**DPC Formation with DNA Glycosylases—**250/C was incubated with DNA glycosylases from *E. coli* (Endo III, Endo VIII, Fpg, and AlkA) and mammalian (hNTH1, hOGG1, and mMPG) for up to 1 h. The molar ratio of 250/C:DNA glycosylase was 1:425–550 in the experiments. Unlike histone and HMG protein, Endo VIII, Fpg, AlkA, and hOGG1 formed DPCs very rapidly (Fig. 4). The DPC band was observed after 10 min of incubation. In contrast, Endo III, hNTH1, and mMPG did not form DPC after 1 h of incubation. With the cross-linked enzymes, the increase in the apparent M_r of DPC relative to free protein was 9000–8800 (Fig. 3A), indicating that DPC was a 1:1 complex of enzyme and 250/C. For Endo VIII, minor DPC species were observed just below the major DPC in the autoradiogram. Because the Endo VIII preparation used here exhibited a CBB-stained single band in protein analysis by SDS-PAGE, the minor DPCs might be due to nicked 250/C, albeit inconclusive. Endo VIII exhibited a very weak incising activity for 250/C (data not shown), which became evident when a large amount of Endo VIII was used as in this experiment. The k_app values for the DNA glycosylases (Endo VIII, Fpg, AlkA, and hOGG1) were two orders of magnitude greater than those for the histone components and HMG protein (Table I), implying distinct mechanisms of DPC formation for the DNA glycosylases and histone/HMG. When the DNA glycosylases (Endo VIII, AlkA, and hOGG1) were inactivated by heat (80 °C for 10 min) prior to the reaction, DPC was not formed after 1 h of incubation (Fig. 5). Conversely, similar heat treatment of histone did not affect DPC formation (Fig. 5). These results indicate that catalytically competent (or properly folded) protein is essential for the rapid DPC formation of DNA glycosylases, whereas proper folding is not essential for the slow DPC formation of histone. Consistent with a previous report (42), Fpg became completely insoluble due to aggregation when treated with heat. Thus, the reaction with heat-denatured Fpg was not possible.

**Sensitivity of DPCs to Proteases and DNases—**To confirm the nature of DPC as a DNA-protein complex, the reaction mixture after incubation of 250/C with protein (histone H2B, Fpg, or Endo VIII) was treated with proteases or DNases. Fig. 6 shows typical results of product analysis by SDS-PAGE. The DPC formed with histone H2B was broken down by trypsin and proteinase K (Fig. 6A), indicating proteolytic cleavage of the associated protein. Conversely, the DPC was resistant to digestion with thrombin that has no recognition sites in histone H2B (X1X2RX3R/KKX4X5X6X7X8 = hydrophobic amino acids and X1 = non-acidic amino acids) (Fig. 6A). Similarly, the DPC was degraded by Dnase I and Exo I (Fig. 6B), showing hydrolysis of the associated DNA. The free DNA was also digested by the enzymes. The DPCs containing Fpg and Endo VIII were also sensitive to digestion with proteases (trypsin and proteinase K (but not to thrombin)) and DNases (Dnase I and Exo I) (data not shown). These results clearly demonstrated that observed DPCs were complexes of DNA and protein.

**Analysis of DPCs Produced by Cell Extracts—**Nuclear extracts from HeLa cells were incubated with 250/C at 37 °C for 1 h. SDS-PAGE analysis of the products revealed two major DPCs (designated as DPC1 and DPC2) (Fig. 7A, lane 2). The formation of DPC1 and DPC2 was rapid, as indicated by their bands appearing after 1 h of incubation. DPC1 and DPC2 were preferentially formed in the presence of 50 and 100 mM KCl compared with in 0 mM KCl (data not shown). The apparent M_r values for DPC1 and DPC2 were 49,000 and 77,000, respectively, suggesting that the original M_r values of cross-linked proteins were ~41,000 and 69,000, respectively. The gel mobility of DPC1 was slightly lower than that of DPC formed between hOGG1 (isoform 1a) and 250/C (Fig. 7A, lane 8, DPC-hOGG1(1a)). As for purified DNA glycosylases (Fig. 5), the formation of DPC1 and DPC2 was sensitive to heat treatment so that virtually no DPC formation was observed when the cell

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**Fig. 3.** Determination of apparent M_r values of free proteins and their DPCs. A, the indicated histone components or HMG protein were incubated with 250/C, and products were separated by SDS-PAGE as shown in Fig. 2. The gel mobilities of free proteins and their DPCs were determined from the CBB-stained bands and autoradiogram, respectively. The mobilities of free histones and DPCs are plotted on the M_r calibration curve obtained with M_r standards (phosphorylase (M_r = 97,400), albumin (66,267), aldolase (42,400), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and lysozyme (14,400)). Symbols: ○, free histone, HMG protein, and single-stranded 250/C; ●, DPCs formed with 250/C (also indicated by asterisks); and ●, M_r standards. B, the gel mobilities of indicated DNA glycosylases and their DPCs are plotted on the M_r calibration curve obtained with M_r standards. Symbols: ○, free DNA glycosylases and single-stranded 250/C; ●, DPCs formed with 250/C (also indicated by asterisks); and ●, M_r standards.

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The data for HMG were calculated using the molecular weight and amino acid composition (Arg (AAD4168). Because there is no report on full-length bovine histone H1, the data for human histone H1c (NP 005311) were used for calculation.

Although histones H2B and H3 contained other histone components as impurities (see Fig. 2b), the data were calculated as pure proteins. The hourly yield (i.e. concentration) of cross-link products. The values were calculated from the data obtained at 12 and 24 h for histones and HMG; 10, 30, 45, and 60 min for DNA glycosylases; 6 and 12 h for N-acetylated amino acids; and 1, 2, and 4 min for polyamines.

To elucidate the amino acid residue involved in DPC formation in proteins, 20 free amino acids were incubated with Oxa—Lys) of HMG-1.

Comparison of the Reactivities of Amino Acids and Amines to Oxanine

-aldehyde Lys 1000 2 0.019 1.9
-aldehyde Arg 1000 2 0.022 2.2
-
Acetyl Lys 1000 2 0.019 1.9
-
Acetyl Arg 1000 2 0.022 2.2
-

The extent of the inhibition was dependent on the concentration of oxanine (25O was 5-labeler with 32P) at 37 °C for 1 h (DNA glycosylases) or 36 h (histone). Products were separated by SDS-PAGE. Autoradiograms of the SDS-gels are shown, and the bands of free DNA and cross-linked products are indicated.

Extract was treated at 80 °C for 10 min prior to the reaction (Fig. 7A, lane 3). When the cell extract was incubated with single-stranded 25O, DPC formation was not observed either (data not shown). Thus, native protein and duplex DNA were indispensable for DPC formation with the cell extract.

The $M_r$ of a protein involved in DPC1 was ~41,000, which was slightly larger than hOGG1 (isofrom 1a, $M_r$ = 38,782) used as a marker. However, hOGG1 has several alternative splicing isoforms, some of which (isofroms 1c (predicted $M_r$ = 45,760), 2a (47,236), 2b (39,729), and 2d (40,094)) are larger than isoform 1a (38,782) (43, 44). Thus, a large isoform of hOGG1 could give rise to DPC1. To clarify whether this was the case, the cell extract was treated with anti-hOGG1 antibody to deplete hOGG1. When 25X/C containing 8oxoG was incubated with the antibody-treated cell extract, incision activity for 25X in the extract, the hallmark of hOGG1 activity, was inhibited (Fig. 7, B and C). The extent of the inhibition was dependent on the amount of the antibody. In contrast, depletion of cellular hOGG1 by the antibody had no effect on the formation of DPC1 (and DPC2) (Fig. 7, A (lanes 5–7) and C), indicating that hOGG1 was not involved in the formation of the observed DPCs.

Comparison of the Reactivities of Amino Acids and Amines to Oxanine—To elucidate the amino acid residue involved in DPC formation in proteins, 20 free amino acids were incubated with

**Table I**

| Protein/amine | Protein/amine $^a$ | 25°C | Cross-link product$^c$ | $k_{app}^d$ | Relative $k_{app}^e$ | Arg + Lys$^f$ |
|---------------|-------------------|------|----------------------|----------|------------------|------------|
|               | $[^{31}S]$        | $h_{app}^*$ | $\text{nmol}^{-1}\text{h}^{-1}$ | $\text{nmol}^{-1}\text{h}^{-1}$ |            |            |
| Histone H1    | 6.0               | 4     | 0.0088               | 1.5 x 10^3 | 79               | 65         |
| Histone H2A   | 9.6               | 4     | 0.019                | 2.0 x 10^3 | 110              | 26         |
| Histone H2B   | 9.7               | 4     | 0.038                | 3.9 x 10^3 | 210              | 28         |
| Histone H3    | 8.7               | 4     | 0.022                | 2.5 x 10^3 | 130              | 31         |
| Histone H4    | 12                | 4     | 0.056                | 4.7 x 10^3 | 250              | 25         |
| HMG           | 2.7               | 4     | 0.010                | 3.7 x 10^3 | 190              | 51         |
| Endo VIII     | 2.2               | 4     | 1.0                  | 4.5 x 10^3 | 24000            | 30         |
| Fpg           | 2.2               | 4     | 1.6                  | 7.3 x 10^3 | 35000            | 35         |
| AlkA          | 2.1               | 4     | 0.27                 | 1.3 x 10^3 | 6800             | 28         |
| hOGG1         | 1.7               | 4     | 0.64                 | 3.8 x 10^3 | 20000            | 39         |
| N-acetyl Lys  | 1000              | 2     | 0.022                | 2.2 x 10   | 1.2              | 1          |
| N-acetyl Lys  | 1000              | 2     | 0.019                | 1.9 x 10   | 1.0              | 1          |
| Spermidine    | 1000              | 2     | 16                   | 1.6 x 10^4 | 840              | —          |
| Spermine      | 1000              | 2     | 15                   | 1.5 x 10^4 | 790              | —          |

$^a$ The molecular weight and amino acid composition (Arg + Lys) of proteins were taken from the GenBank database: H2A (accession number: HSBO2A), H2B (HSBO22), H3 (HSBO3), H4 (HSBO4), HMG (S01947), Endo VIII (AAC45355), Fpg (X06036), AlkA (K02498), and hOGG1 (AAD4168). Because there is no report on full-length bovine histone H1, the data for human histone H1c (NP 005311) were used for calculation.

$^b$ The hourly yield (i.e. concentration) of cross-link products. The values were calculated from the data obtained at 12 and 24 h for histones and HMG; 10, 30, 45, and 60 min for DNA glycosylases; 6 and 12 h for N-acetylated amino acids, and 1, 2, and 4 min for polyamines.

$^c$ $k_{app} = [\text{cross-link product/([25O/C] x [protein (amine)])}]$.

$^d$ Ratios of $k_{app}$ relative to that of N-acetylated Lys.

$^e$ The number of Arg and Lys residues per molecule.
Fig. 6. Digestion of DPC containing histone H2B with proteases and DNases. 25O/C and histone H2B were incubated as described in Fig. 2, and an aliquot of the reaction mixture was further treated with proteases (A) or DNases (B). Products were analyzed by SDS-PAGE. The left-most and second lanes for each enzyme show free DNA and a reaction mixture without enzyme digestion, respectively. In A, the sample was digested with trypsin or proteinase K (both 0.0001, 0.001, 0.01, and 0.1 unit from left to right lanes) for 10 min, or with thrombin (0.01, 0.1, 1, and 10 units) for 1 h. In B, the sample was digested with DNase I (0.1, 0.2, 0.5, and 1 unit from left to right lanes) or Exo I (1, 2, 5, and 10 units) for 1 h. 

25O/C at 37 °C for 48 h (molar ratio of 25O/C:amino acid = 1:4 × 10^5), and products were analyzed by denaturing PAGE. Free Cys, Lys, Arg, and His formed adducts with Oxa in 25O/C (Fig. 8A). 25O containing the amino acid adduct migrated slower than intact 25O in denaturing PAGE. The adduct formation occurred with 25O/C but not 25O/C containing G in place of Oxa at the same position (Fig. 8B). Because the free N°-amino group of Cys, Lys, Arg, and His is not available in the protein for the reaction due to amide bond formation, the reaction with N°-acetylated derivatives of the amino acids were further examined. PAGE analysis revealed that the N°-acetylated derivatives of Lys and Arg but not those of Cys and His reacted with 25O/C (data not shown). These results indicate that the side chains of Lys (–(CH2)4NH2) and Arg (–(CH2)4NH=NH(NH2)2) in protein are possibly involved in DPC formation. The reactivity of the side chains of Lys and Arg for 25O/C was comparable when assessed using N°-acetylated derivatives of Lys and Arg (Fig. 9).

Because a subgroup of free amino acids formed adducts with 25O/C, the reaction with monoamines and biologically relevant polyamines was examined further. The tested monoamines were propylamine (NH2CH2CH2CH2NH2) and dipropylamine (NH(CH2)4NH(CH2)2NH2), and polyamines were spermidine (NH2(CH2)4NH=NH(NH2)2), and putrescine (NH2(CH2)4NH2). The ratio of amines:25O/C for the reaction was the same as that for amino acids (molar ratio of 25O/C:amine = 1:4 × 10^5). Spermidine and spermine reacted with 25O/C very rapidly to form adducts (Fig. 8B), with most of the 25O/C converted to the adduct after 15 min of incubation (Fig. 9). The k app values of spermidine and spermine were 800-fold higher than those of N°-acetylated derivatives of Lys and Arg (Table 1). Although putrescine also formed an adduct, the reactivity for 25O/C was comparable to those of N°-acetylated derivatives of Lys and Arg (data not shown). The monoamines (propylamine and dipropylamine) did not form adducts even after 48 h of incubation. Accordingly, the reactivity of the amines for Oxa varied markedly and appeared to be dependent on the number of amino groups (or positively charged groups) in the molecule (polyamine > diamine >

Fig. 7. Analysis of cross-link products formed between Oxa and HeLa cell extracts. A, 25O/C containing Oxa (25O was 5°-labeled with 32P) was incubated with the cell extract (lanes 2 and 4), heat-treated cell extract (lane 3), and anti-hOGG1 antibody-treated cell extract (lanes 5–7) at 37 °C for 1 h. Products were separated by SDS-PAGE. The autoradiogram of the SDS-gel is shown. Lane 8 shows a cross-link product (DPC1) prepared by the reaction of 25O/C with hOGG1 isoform 1a. The amount of antibody added to the cell extract was 2.5 µl, but serially diluted as follows: lane 5, 1/100 dilution; lane 6, 1/10 dilution; and lane 7, no dilution. The bands of free DNA, two major cross-link products formed by the cell extract (DPC1 and DPC2), and DPC-hOGG1(1a) are indicated. B, 25XC containing 8-oxoG (25X was 5°-labeled with 32P) was incubated with the cell extract (lane 2) or anti-hOGG1 antibody-treated cell extract (lanes 3–5) at 37 °C for 1 h. The cell extract was pretreated with the antibody as described in A, and the products were separated by 16% denaturing PAGE. The autoradiogram of the gel is shown. C, the amount of DPC1 formed with 25O/C (A) and that of the nicked product formed with 25X/C (B) are plotted against the amount of anti-hOGG1 antibody used for treatment of the cell extract. The data are standardized to those without antibody treatment. Open bar, DPC1 formed with 25O/C; closed bar, nicked product formed with 25X/C.

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monoamine (reaction not observed). With the exception of Cys, this was also the case for free amino acids. Lys, Arg, and His bearing a positively charged side chain reacted with Oxa but other amino acids did not under the present conditions (see above). It is tempting to speculate that electrostatic interactions between the negatively charged DNA backbone and the positive charge in the polyamines or amino acid side chains facilitate the association of DNA and nucleophilic molecules to form adducts.

**DISCUSSION**

NO and nitrous acid induce deamination of C and A, resulting in U and Hx, respectively. To avoid genotoxic effects of the aberrant bases, U and Hx are removed from DNA by uracil-DNA glycosylase and methylpurine-DNA glycosylase, respectively, both in eukaryotic and prokaryotic cells (45, 46). NO and nitrous acid also induce deamination of G, yielding Xan and Oxa. However, the cellular repair mechanism of Xan and Oxa has been clarified only partially (47). Although unrepaired Xan and Oxa lesions can exert genotoxic effects by directly affecting DNA replication (19, 48, 49) or reducing the stability of duplex DNA (20), in this study we have demonstrated a novel genotoxic mechanism of Oxa that involves DPC formation. Oxa in DNA reacted with nuclear proteins such as histone, HMGB protein, and DNA glycosylases to form DPCs (Figs. 2 and 4).

The nature of the DPCs as complexes of DNA and protein was further confirmed by the protease and DNase treatment (Fig. 6). Small amine molecules such as spermine, spermidine, and a subgroup of amino acids also reacted with Oxa to form adducts (Fig. 8). The formation of DPCs (DPC1 and DPC2) was also observed when 25O/C containing Oxa was incubated with the nuclear extract from HeLa cells (Fig. 7A). Although purified hOGG1 reacted with 25O/C (Fig. 4), DPC1 and DPC2 formed with the HeLa cell extract did not contain the possible isoforms of hOGG1. However, formation of DPC1 and DPC2 was rapid and sensitive to heat treatment and required duplex DNA. Thus, like DNA glycosylases, the proteins involved in DPC1 and DPC2 appear to interact with Oxa in a damage-specific manner.

DPCs are induced by a number of chemical (e.g. aldehydes, nitrous acid, and metal compounds such as arsenite, nickel, and chromate) and physical agents (e.g. UV light and ionizing radiation) (Refs. 50 and 51 and references cited therein). These agents are mutagenic, and many of them are suspected or known carcinogens, although the mechanistic link between DPCs and carcinogenesis has not been fully elucidated. However, it can be readily inferred from their bulky nature that DPCs formed by NO (in this study) and those mentioned above raise a barrier to progression of a DNA replication fork or reduce the fidelity of DNA synthesis and consequently lead to chromosomal aberrations (52). Consistent with this, only those aldehydes with a specific DPC-forming ability disrupt SV40 replication (53). Together with NO and ONOO -, hypochlorous acid (HOCl) is an important component of host defenses against infection and inflammatory responses (54). HOCl formed by the myeloperoxidase-H2O2-Cl- system of phagocytes generates DPCs between single-stranded DNA-binding protein and a single-stranded oligonucleotide (dT40) (55). DPCs are also formed in E. coli exposed to HOCl (55). Accordingly, DPCs formed by NO and HOCl may help elucidate the molecular mechanism of genotoxic and cytotoxic effects in inflammatory responses.

The comparison of $k_{app}$ values has revealed that a subset of DNA glycosylases (hOGG1, Fpg, AlkA, and Endo VIII) reacts with Oxa much more rapidly than histone and HMG protein. The content of Arg and Lys potentially involved in DPC formation differs depending on the proteins, but the difference appears too small to fully account for the large difference in $k_{app}$ for the DNA glycosylases and histone/HMG protein (Table I). Also, native (or active) structures were essential for rapid DPC formation with the DNA glycosylases, whereas the rate of DPC formation with histone was independent of heat denaturation (Fig. 5). These results strongly suggest that slow DPC formation with histone and HMG protein occurs via nonspecific DNA-protein interactions, whereas fast DPC formation with the DNA glycosylases occurs via damage-specific interactions. hOGG1, Fpg, AlkA, and Endo VIII use a base-flipping mecha-
zymes by covalent trapping has a dual biological effect: DPC between DNA and UvrA/UvrB proteins involved in nucleotide and is used as an anticancer drug, also induces cross-links between aliphatic molecules.

It should be noted that, although the value of $f_{amp}$ is useful for elucidating the mechanistic aspect of DPC formation, this parameter does not necessarily correlate with the biological significance of a particular type of DPC. Histone and HMG protein (a major non-histone protein) are abundant in eukaryotic cells and are associated with DNA to maintain chromatin structure or to coordinate transcription. A high abundance of the proteins and the constant association with DNA may overwhelm the low reactivity to Oxa and, consequently, result in a significant amount of DPCs. Alternatively, constant damage surveillance of DNA glycosylases (62, 63) and DNA polymerase -acylurea in Oxa (Fig. 9). Recently, it has been shown that a covalent amide bond with a proximal amino acid such as Arg or Lys in the active site pocket (69).

unlabeled derivatives suggested the formation of a covalent amide bond or to coordinate transcription. A high abundance of the enzymes for Oxa is virtually negligible leading to a significant amount of DPCs. Thus, evaluation of the mechanistic aspect of DPC formation, this parameter does not necessarily correlate with the biological significance of a particular type of DPCs (and amine adducts) must await quantification of individual types of DPCs and amine adducts in cells exposed to NO or nitric acid.

Analysis of the reactivity with amino acids and their acetylated derivatives suggested the formation of a covalent amide bond between the side chain of Arg or Lys in protein and O-acrylurea in Oxa (Fig. 9). Recently, it has been shown that a covalent amide bond is formed between 2-deoxyribo- lactone and sugar lesions, respectively, they have a lactone structure in common that is prone to react with nucleophilic molecules.

cis-Platinum, which is known to form DPC and is used as an anticancer drug, also induces cross-links between DNA and UvrA/UvrB proteins involved in nucleotide excision repair (66). Thus, irreversible inhibition of repair enzymes by covalent trapping has a dual biological effect: DPC formation in general and suicide of DNA repair enzymes in particular. Both could pose a threat to the genetic and structural integrity of DNA, hence initiating a carcinogenic process.

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mMPG that recognizes methylated purines and Hx (61), DPC formation for elucidating the mechanistic aspect of DPC formation, this parameter does not necessarily correlate with the biological significance of a particular type of DPCs (and amine adducts) must await quantification of individual types of DPCs and amine adducts in cells exposed to NO or nitric acid.

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DNA-Protein Cross-link Formation Mediated by Oxanine: A NOVEL GENOTOXIC MECHANISM OF NITRIC OXIDE-INDUCED DNA DAMAGE
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