Quenching of DNA Cross-Link Precursors of Chloroethylnitrosoureas and Attenuation of DNA Interstrand Cross-Linking by Glutathione

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ABSTRACT

Interstrand DNA cross-linking is essential for the antitumor activity of chloroethylnitrosoureas (CENUs). The critical cross-links have been proposed to involve a rapid O^6-guanine chloroethylation on one DNA strand, followed by a rearrangement of the O^6-(2-chloroethyl)guanine and slow alkylation of the second DNA strand. In view of the relative intracellular abundance of glutathione (GSH) and nucleophilicity of its thiolate ion, the ability of GSH to react with and to inactivate 2-chloroethylated DNA and the possibility that this interaction decreases net DNA cross-linking by CENUs were investigated. Chloroethylated calf thymus DNA was reacted with GSH, the DNA was precipitated and redissolved, and subsequent DNA interstrand cross-linking was determined. The DNA cross-link index was compared for both GSH-treated and 2-chloroethylated untreated DNA. Simultaneously, Col E, plasmid DNA was chloroethylated and reacted with GSH, and the extent of DNA interstrand cross-linking was determined by agarose gel electrophoresis and compared with controls. The results show both a time- and GSH concentration-dependent quenching of chloroethylated DNA, with a corresponding decrease in the DNA cross-link index. Using [methyl-^3H] GSH, it was also demonstrated that 56% of the total GSH was bound to quenched 2-chloroethylated Col E DNA and 25% to quenched 2-chloroethylated calf thymus DNA. GSH binding to cross-linked DNA and native DNA was insignificant. It is concluded that, in addition to direct inactivation of reactive cytotoxic CENU species, GSH may also modulate cellular response to CENUs by quenching chloroethylated DNA, thereby decreasing the formation of potentially lethal DNA cross-links.

INTRODUCTION

The CENUs belong to the clinically most active group of agents used in brain tumor chemotherapy (1). Early studies (2, 3) have demonstrated that these compounds undergo spontaneous hydrolytic decomposition, both in aqueous buffers and under intracellular pH conditions, to yield, via a diazonium hydroxide, bifunctionally alkylating chloroethylcarbanion ions and carbamoylating organic isocyanates. Although various nucleophilic sites on cellular biomolecules can be attacked by both CENU decomposition products, evidence (4, 5) strongly suggests that DNA interstrand cross-linking by the chloroethylcarbanion ions is the critical cytotoxic lesion induced by CENUs. Presumably, this cross-linking of the two complementary DNA strands, if not repaired, incapacitates the DNA as a template for semiconservative DNA replication. There is evidence (6) to support the formation of the cytotoxic DNA interstrand cross-link to involve an initial chloroethylation of the O^6-position of guanine on one DNA strand, followed by an intramolecular rearrangement and alkylation of cytosine at the N^7-position on the opposing DNA strand, resulting in a 1-(N^7-deoxyctydyl), 2-(N^4-deoxyguanosyl)ethane interstrand cross-link. The initial guanine O^6-chloroethylation (monooadduct formation) is very rapid and occurs within minutes while the cross-linking reaction requires several hours (4, 5). Consequently, the extent of cross-linking can be affected by molecules that can either dealkylate the monoadduct or alternatively react with it and thereby prevent it from reacting with cytosine to form a G-C cross-link. The tripeptide gamma-L-glutamyl-L-cysteinyl-glycine (GSH) is abundantly present in cells in concentrations up to 10 mm (7) and can dissociate at physiological pH to yield a thiolate nucleophile. In this study, the ability of GSH to react with CE-DNA and consequently quench it as a precursor of DNA interstrand cross-linking was investigated using both calf thymus DNA and the Col E, plasmid DNA. Plasmid DNA was used in addition to eukaryotic DNA primarily to permit the use of agarose gel electrophoresis as one of the techniques with which to quantify DNA interstrand cross-linking. The data demonstrate that treatment of CE-DNA with GSH results in a dose-dependent and GSH treatment time-dependent attenuation of DNA interstrand cross-linking of the CE-DNA. It is postulated that the reaction of GSH with chloroethylated (cross-link precursor) DNA may represent an important molecular mechanism by which cells can be protected from the cytotoxic action of CENUs.

MATERIALS AND METHODS

Biochemicals. Calf thymus DNA, ethidium bromide, glutathione, and other chemicals and reagents were purchased from Sigma, St. Louis, MO. BCNU was purchased from Bristol Myers Co., New Haven, CT.

Col E, plasmid DNA was prepared by chloramphenicol amplification of the plasmid in Escherichia coli JC 411 (thy^-, met^-, leu^-, his^-, ang^) followed by lysozyme/Triton X-100 lysis and CsCl centrifugation as previously described (8).

Preparation of Chloroethylated Calf Thymus DNA. Fifty µg of calf thymus DNA in TE buffer were reacted with 2 mM BCNU for 15 min at 45°C. Control experiments showed that there was minimal DNA cross-linking during this period. The largely CE-DNA was precipitated with 3 volumes of ethanol containing 0.25 M sodium acetate for 1 h at -70°C. The DNA was precipitated at 10,000 x g for 15 min, washed twice with 80% ethanol (residual ethanol removed under vacuum), and redissolved in TE buffer. CE-DNA thus prepared was placed in ice and used immediately or stored at -70°C until used. Control DNA samples without BCNU pretreatment were similarly prepared.

Reaction of DNA Monoadducts with GSH. To aliquots of CE-DNA, corresponding to 40 µg of DNA, were added 50 µl of freshly prepared GSH solutions to achieve GSH concentrations of 0 to 5 mM. The mixtures were then incubated at 37°C for 3 h, and the DNA was precipitated and washed as described earlier. After dissolution in TE buffer, the DNA solutions were incubated for a further 6 h to allow cross-linking of residual, uncleaved CE-DNA to occur. The amount of DNA interstrand cross-linking was then determined using the ethidium bromide binding assay described below. In order to determine the kinetics of CE-DNA quenching by GSH, CE-DNA was incubated at 37°C. DNA interstrand cross-linking was then measured.

Measurement of CT-DNA Interstrand Cross-Linking. The method used to quantify DNA interstrand cross-linking was modified from that described by Morgan and Petkau (9) and Brent (10). The technique is based on the observation that native double helical DNA binds EB efficiently and, in contrast, denatured DNA binds EB poorly. If cross-linked DNA is denatured, e.g., by heat, and then renatured by rapid
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cooling, then because the bases are kept in register by the interstrand cross-links, there is a quick reversion to the double helical structure with a concomitant recovery in EB binding. On the other hand, non-cross-linked DNA, upon heat denaturation followed by cooling, does not readily reanneal to reform the double helix and, consequently, EB binding remains low. The extent of EB binding is determined fluorometrically. In this study, 3 ml of a 10-μg/ml EB solution in EB buffer consisting of 20 mM K2HPO4, and 0.4 mM EDTA were added to the DNA solution. The mixture was then heated to 100°C for 5 min and then cooled rapidly to room temperature. The fluorescence with and without the heating and cooling cycle was determined using a Perkin-Elmer LS 50 fluorometer at 530-nm excitation and 590-nm emission wavelengths.

Assuming that DNA cross-links are randomly formed, the average number of cross-links per DNA molecule (Nₓ) was calculated using the formula (10)

\[ N_x = -\ln X \]

where X is the fraction of EB fluorescence retained after the denaturation/denaturation cycle.

Electrophoretic Determination of Col E, DNA Cross-Linking. This assay was modified from that described by Robbins et al. (11). In an agarose gel, native negatively supercoiled circular Col E, plasmid DNA moves electrophoretically as what is customarily referred to as Form I, and after denaturation the compact, randomly coiled DNA moves faster as Form IV (Fig. 1). Thus, if samples of native Col E, DNA, with and without cross-linking are denatured and then brought to renaturing conditions, the cross-linked DNA will renature rapidly and move electrophoretically as Form I, while the randomly coiled uncross-linked DNA will move predominantly as Form IV.

In this study, samples of approximately 1 μg of Col E, DNA, as required, were denatured by adding 12.5 μl NaOH to achieve 0.5 M NaOH. The final reaction volume of 200 μl was maintained at room temperature for 5 min and then neutralized with a 15- to 20-μl potassium acetate solution (containing 3 M K+ and 5 M acetate). The DNA was precipitated with 3 volumes of ethanol at -20°C overnight. After centrifugation at 10,000 rpm for 5 min, the pelletted DNA was carefully dried and dissolved in 20 μl of Tris-EDTA buffer and 5 μl of a solution of 20% Ficoll and 0.05% bromophenol blue. Samples were electrophoresed in 1.2% agarose in 80 mM Tris-phosphate-2 mM EDTA buffer at 40 V overnight. At the end of the run, the gels were stained with 0.5 μg/ml of ethidium bromide in running buffer and photographed under UV (302 nm) light.

Quenching of Chloroethylated Col E, DNA. CE Col E, DNA was prepared by treating 10 μg of DNA with 2 mM BCNU in potassium phosphate buffer, pH 7.4, for 10 min at 37°C. Three volumes of ethanol were then added after 2 h at -70°C, and the DNA was pelleted at 10,000 rpm, washed twice with cold 80% ethanol, and redissolved in 20 mM potassium buffer, pH 7.5. The CE-Col E, DNA thus prepared was kept on ice and used immediately or was stored at -70°C until used. To determine GSH quenching of the chloroethylated DNA cross-link precursor, duplicate tubes containing CE-DNA (2.5 μg) were treated with 0.5 mM and 2.5 mM GSH, respectively. Control tubes received buffer without GSH. Simultaneously, tubes were set up containing 2.5 mM GSH: and (a) Col E, DNA that had been treated with 2 mM BCNU and allowed to cross-link over 18 h at 37°C; (b) native Col E, DNA that had not reacted with BCNU; and (c) denatured Col E, DNA. The tubes were all incubated for 6 h, and the DNA interstrand cross-linking was determined by agarose gel electrophoresis as described earlier. Control of native, denatured, and cross-linked Col E, DNA without GSH treatment was also run.

Quantification of GSH Binding to Chloroethylated DNA. To quantitate the extent of GSH binding to DNA cross-link precursors, 1H-labeled GSH was used to perform the quenching experiments essentially as described above. Twenty-five μg of CE calf thymus DNA in 50 μl of TE buffer were reacted with 10 μl of [glycine-3H]GSH for 9 h. The DNA was precipitated with sodium acetate/ethanol as described earlier, and the precipitated DNA was washed twice with 80% ethanol to free it of unbound [3H]GSH. The pellet was then taken up in scintillation cocktail, and the radioactivity was counted. Cross-linked DNA and DNA that had not reacted with BCNU were similarly treated with [3H]GSH and used as controls. The amount of [3H]GSH bound to the DNA was determined as the fraction of the total radioactivity (cpm) present in the [3H]GSH used to quench the DNA.

A second series of quenching experiments were performed using Col E, plasmid DNA. The conditions of quenching were similar to those described earlier, except that [glycine-3H]GSH was used instead of cold GSH. After electrophoresis in 1.2% agarose and photodocumentation of the DNA bands, the bands corresponding to Forms I (sicked circular DNA) and IV were cut out and dissolved in scintillation cocktail, and the radioactivity was counted by liquid scintillation.

RESULTS

Dose-Response and Kinetic Studies on GSH Quenching of Calf Thymus CE-DNA. Fig. 1 demonstrates the relationship between GSH concentration and the extent of calf thymus CE-DNA quenching, measured as the decrease in the DNA interstrand cross-link index. As shown by the curve, increasing GSH concentrations resulted in a linear decrease in the DNA cross-link index with a leveling off after about 0.5 mM GSH. Maximum decrease in DNA cross-linking was 53% at 2.5 mM GSH. The kinetics of quenching of CE-DNA at a constant GSH concentration of 2.5 mM is shown in Fig. 2. The curve suggests pseudo-first-order kinetics for CE-DNA quenching, with most of the quenching occurring within the first 6 h.

Quenching of Chloroethylated Col E, Plasmid DNA. Fig. 3 shows the electropherograms (1.2% agarose gel) of Col E, plasmid DNA in the cross-link precursor quench studies. Native DNA (Lane I) under the experimental conditions moved as Form I (with some nicked circles appearing as Form II), while...
denatured DNA (Lane 2) moved as the randomly coiled, compact Form IV. There was no change in the pattern of native or denatured DNA treated with 2.5 mM GSH (Lanes 3 and 4). Lane 5 contained chloroethylated DNA that had been treated with 2.5 mM GSH, immediately frozen, and thawed just before being electrophoresed. Most of this DNA is present as the non-cross-linked Form IV, and only a small amount of Form I was observed. Lane 6 shows chloroethylated DNA that was allowed to go on to cross-link and then treated with 2.5 mM GSH. Most of the DNA in this lane is Form I, with hardly any Form IV. Lanes 7 and 8 represent chloroethylated DNA that had been quenched with 2.5 and 0.5 mM GSH, respectively, and then incubated for a further 12 h to allow for cross-link formation. In both lanes, there is an increase in the Form IV (non-cross-linked DNA) and a decrease in Form I (band containing cross-linked DNA). CE-DNA that had been quenched with 2.5 mM GSH (Lane 7) showed a greater amount of non-cross-linked DNA than that quenched with 0.5 mM GSH (Lane 8).

Binding of Radiolabeled GSH to Quenched DNA. The results of these studies are summarized in Table 1 (CT-DNA and Col E, DNA). As shown in Table 1, 24.9% of the total radioactivity was recovered in the quenched CE-DNA, with only 4.3% and 2.4% in the cross-linked and nonalkylated DNA, respectively. Table 1 also shows that, after quenching chloroethylated Col E, plasmid DNA with [3H]GSH, about 56% of the DNA-bound radioactivity was found in the Form IV (non-cross-linked DNA) band. The Form I:Form IV ratio of bound radioactivity in cross-linked DNA was 1:4.1. For native Col E, DNA to which [3H]GSH was added and immediately frozen until electrophoresis, the Form I:Form IV ratio of bound radioactivity was 1.5:1, while for [3H]GSH-quenched CE-DNA, the ratio was 3:1.

The binding of radiolabeled GSH to quenched DNA was further studied with purified and cellular DNA that many nucleophilic sites on DNA can be alkylated by the CENUs (3, 4, 21). Using N-ethyl-nitrosourea, it has been shown that the phosphates of the phosphodiester bond and the N7 position of guanine together account for about 70% of the total DNA alkylation (22). These alkylated sites are unstable (22), and the N7-alkylguanine DNA is readily depurinated. Nonetheless, it is not unlikely that, under the experimental conditions of this study, some amount of the reaction of GSH with CE-DNA (as demonstrated by the binding of [3H]GSH to CE-DNA) could still be occurring at the phosphodiester and N7 sites. However, the results clearly demonstrate that not only is GSH bound to quenched CE-DNA, but also that there is a very significant decrease in subsequent DNA interstrand cross-linking by the quenched CE-DNA. This is evidence that a reaction between the DNA cross-link precursor, O6-chloroethylguanine, and GSH does indeed take place. This conclusion is further supported by the fact that, in control experiments, GSH bound proportionately less to cross-linked DNA than that quenched with 0.5 mM GSH (Lane 8). It should be mentioned that, in the GSH-binding studies using Col E, DNA, some amount of nonspecific "binding" of GSH to DNA was observed as radioactivity bound to both nicked plasmid and cross-linked DNA.
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Fig. 4. Proposed mechanism for quenching of chloroethylated DNA and attenuation of DNA interstrand cross-linking by GSH.

Based on the postulated mechanism of DNA interstrand cross-linking by CENU (6), the quenching of CE-DNA is proposed to occur as outlined in Fig. 4. This mechanism suggests that, after an initial chloroethylation and intramolecular rearrangement, the cross-link precursor reacts with GSH to form an S-substituted glutathione. This is similar to the reaction recently proposed by Brent (23) to occur between the DNA cross-link precursor of BCNU and the DNA repair enzyme, O\textsuperscript{6}-alkylguanine DNA AT, an AT reaction that is distinct from the transfer of the alkyl group from the O\textsuperscript{6}-alkylguanine to the AT protein (10, 24). The quenching of CE-DNA by GSH observed in this study may represent a more generalized ability of GSH to react with DNA cross-link precursors of bifunctional alkylating agents. Indeed, it has been demonstrated (25) that GSH quenches precursors of DNA cross-links generated by CDDP. Hydrolysis of DNA that had been monoadducted with CDDP and subsequently quenched with GSH allowed identification of a putative glutathione-platinum-DNA adduct (26). Efforts are ongoing in our laboratories to identify the adduct postulated to be a product of GSH quenching of chloroethylated DNA. The reaction of GSH with DNA interstrand cross-link precursors as observed in this study may, if demonstrated to occur intracellularly, represent an important, hitherto unrecognized molecular mechanism by which cells may modulate their susceptibilities to CENUs, i.e., become CENU resistant. It may be postulated that a specific GST (glutathione-S'-transfase) will catalyze this conjugation, as has been demonstrated for the formation of S-[2-(N\textsuperscript{7}-guanyl)ethyl]glutathione upon reacting 1,2-dibromethane with DNA and GSH (27). If confirmed, the extent of CENU resistance that will be attributable to this mechanism of monoadduct quenching will consequently depend not only on the intracellular GSH concentration, but also upon the level of this specific GST present in the cells.

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