Complexing of Amino Acids to DNA by Chromate in Intact Cells

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Using ophthaldehyde (OPT) fluorescence, the amino acids associated with DNA were studied following exposure of intact Chinese hamster ovary cells to chromate. Rigorous extraction with EDTA, acid, or base was required to release the amino acids cross-linked to the DNA isolated from control or chromate-treated cells by standard procedures (i.e., proteinase K, phenol, etc.). Amino acids resisting extraction from DNA were not studied since analysis was limited to those that could be released by these procedures. There was a chromate dose-dependent increase in amino acids complexed with the DNA that could be released by EDTA, acid, and base, and these amino acids were separated by HPLC and identified. Substantial increases in cysteine, glutamine, glutamic acid, histidine, threonine, and tyrosine were found as a function of increasing concentrations of chromate. There was also a time-dependent increase in complexing of these amino acids to the DNA by chromate. The amino acids found complexed to DNA in intact cells by chromate were thought to originate from reactions of free amino acids or small peptides with the DNA rather than being proteolytic products derived from larger proteins that were cross-linked to the DNA. This was supported by a number of experiments: a) free amino acids or bovine serum albumin (BSA) were cross-linked by chromium to DNA in vitro and the DNA was isolated by standard procedures. With BSA, few amino acids were found cross-linked to the DNA, but with free amino acids, numerous amino acids were associated with DNA; b) when radiolabelled threonine complexed to the DNA was examined in the absence and presence of a protein synthesis inhibitor, there was substantial stimulation of the threonine complex to DNA by chromate when protein synthesis was inhibited with cyclohexamide; c) there were substantial increases in amino acids associated with DNA isolated without protease. Not only does the cross-linking of amino acids to DNA represent a new type of lesion to study in intact cells but it may also be a useful biomarker of human exposure to cross-linking agents. — Environ Health Perspect 102(Suppl 3):251–255 (1994).

Key words: cysteine, histidine, glutamine, HPLC, DNA extraction

Introduction

Even the most highly purified DNA has residual amino acids associated with it that can be detected by extraction with strong acids, bases, or other rigorous extraction procedures (1,2). These residual amino acids are thought to have been part of the original chromatin-scaffold proteins or other tightly bound chromatin proteins (2). A number of proteins are covalently cross-linked to the DNA and these proteins are cross-linked through specific amino acid residues, which should be detected when amino acid–DNA cross-links are examined. An example of one such protein is topoisomerase (3).

Previous studies in our laboratory have utilized DNA–protein cross-links as an indicator of exposure to various chemical agents, such as chromate, nickel, formaldehyde, UV light, etc. (4,5). We have developed assays for detection of DNA-protein complexes (4,6) and proteins associated with DNA (5) have been studied. This type of lesion has been used as a human biomarker of exposure to cross-linking agents (7). However, the ability to extract DNA in the presence of intact proteins is limited, and most known procedures are generally flawed with background problems. DNA isolated by standard proteinase K/phenol/chloroform extraction procedures, however, yielded highly purified DNA with a minimal of background proteins. This DNA also had a minimum of associated background amino acids (2,8).

In the present study, we have attempted to examine whether residual amino acids associated with DNA isolated by the proteinase K method could be used to detect exposure to cross-linking agents. There are substantial increases in residual amino acids associated with DNA found in intact cells treated with a cross-linking agent such as chromate. Direct examination of amino acids by fluorescent derivatives and HPLC separation yielded an accurate assessment of the actual amino acid crosslinked to the DNA.

Materials and Methods

Chinese hamster ovary cells (CHO) were cultured in α-MEM at 37°C, supplemented with 10% fetal bovine serum and antibiotics in an atmosphere containing 95% air and 5% CO₂. Cells were cultivated in 150 mm dishes to approximate 30% confluency. Subsequently, they were treated with various concentrations of K₂CrO₇ ranging from 5 to 100 μM for 24 hr in complete media. Cells were subsequently rinsed with phosphate buffered saline, scraped, collected by centrifugation and lysed in 0.5% SDS solution containing 100 mM sodium chloride and 10 mM HEPES pH 8.0. Cells were concentrated to about 2 to 3 million per ml. Cells were passed through a 25-gauge needle and 25 μg/ml of RNase was added and the homogenate was incubated for 30 min at 37°C. Subsequently, 300 μg/ml of proteinase K was added and the cells were incubated for 3.5 hr at 50°C. This homogenate was extracted three times with phenol/chloroform/isomyl alcohol at a ratio of 25:24:1 using equal volumes. The aqueous phase was subsequently extracted with chloroform/isomyl alcohol at 24:1 using equal volumes. The aqueous phase, which contained the DNA, was made 0.1 M in NaCl two volumes of ethanol were
added and the aqueous phase was allowed to stand overnight at −20°C. DNA was collected by centrifugation for 20 min at 12,000 g and the pellet was rinsed in 70% ethanol. The final DNA pellet was dissolved in 20 mM of HEPES buffer, pH 8.0. DNA was subsequently extracted with either EDTA, NaOH, or sulfuric acid as described in the figure legends.

Amino acids associated with the DNA were quantitated by reaction with the fluorescent dye o-phthalaldehyde (OPT) (9). Sixty milligrams of OPT was dissolved in 1 ml of ethanol, and 40 μl of 2-mercaptoethanol and 1 ml of borate buffer, pH 9.5 were added. Fifty microliters of amino acid solution was reacted with 5 μl of this OPT reagent for exactly 1 min. The reaction was stopped with 5 μl of 50 mM of NaCl, pH 4.5. Total fluorescence was either determined directly using a Perkin-Elmer fluorescent spectrophotometer (Norwalk, CT) or DNA samples were precipitated with ethanol, the supernatant was dried, and the residue dissolved in 15 μl was injected in a HPLC column. Amino acids were separated on Adsorbosphere OPA-US column (Alltech, Dierfield, IL) in a NaCl, H2O buffer, pH 5.7, containing 3% 2-propanol against 100% methanol containing 1.5% 2-propanol for 40 min at a flow rate of 1.5 ml/min (grad: 0–80% methanol for 40 min).

A special procedure was utilized to determine cysteine content associated with the DNA. DNA purified from cells was used for alklylation of cysteinic residues to convert them to S-carboxymethyl cysteine (CMC). Dithiothreitol was added to the DNA to a final concentration of 2 mM. Nitrogen was blown over the surface of the solution and the reaction mixture was incubated at 37°C for 1 hr. Then 4 mM iodoacetic acid and 0.2 N NaOH were added to the solution and nitrogen was again blown over the surface of the solution. Release of amino acids from the DNA and alklylation of released cysteine residues were allowed to proceed in the dark at 37°C for 2 hr. DTT and iodoacetate were not able to agree release cysteine residues from the DNA. Treatment of the DNA with NaOH was required to render thiol groups of cysteine accessible to the reagents for alklylation that allowed us to detect cysteine residues bound to DNA following Cr-treatment of CHO cells.

Experiments to determine complexing of proteins or amino acids with DNA in vitro were performed using calf thymus DNA, standard amino acids, and bovine serum albumin. One hundred micrograms per milliliter DNA was incubated with 0.5 mg/ml BSA or 50 μM each of amino acids in the presence or absence of 100 μM CrCl3 for 24 hr at 37°C as indicated in Figure 6. DNA was purified with the proteinase K/phenol/chloroform procedures as described for CHO cells, except that proteinase K was not applied to the samples of DNA incubated with amino acids.

Results

Figure 1 shows the total fluorescent amino acids that could be extracted from the DNA in untreated or cells treated with 50 or 100 μM chromate. Note the increase of fluorescent derivatives of DNA associated amino acids that was dose-dependent following treatment of cells with increasing concentrations of chromate. The amino acids associated with DNA were separated by HPLC to resolve the individual amino acids contributing to the overall fluorescence.

Figure 2 shows the separation of amino acids present in control or chromate-treated DNA. A number of NaOH-extractable amino acids were increased in their cross-linking compared with control DNA. Particularly striking was the increase in carboxymethylcysteine, glutamic acid, glutamine, and histidine.

Figures 3 and 4 quantitate and compare a number of the amino acid peaks that were resolved by HPLC. Note that the consistency of the method is illustrated by the small standard-error bars and note that NaOH was the most effective agent in releasing amino acids. Figure 3 is a quantitative comparison from both control and chromate-treated cells, while Figure 4 illustrates the cross-linked amino acids relative to control. Glutamic acid and glutathione were more strikingly associated with DNA after chromate treatment. Other amino acids were also increased in their associa-
PRELIMINARY CHARACTERIZATION OF AMINO ACID-DNA ADDUCTS

Figure 3. Quantitation of amino acids associated with DNA. DNA was treated with EDTA, acid, or sodium hydroxide as described in Figure 1. The amino acids were separated with HPLC and several amino acid standards were used to identify and quantitate unknown peaks.

Figure 4. Amino acids associated with DNA following treatment with chromate. CHO cells were treated with 50 or 100 μM potassium chromate for 20 hr in complete MEM. Samples were prepared as described in Figure 1 and amino acids were separated by HPLC as described in Materials and Methods. The ratio of each amino acid amount extracted from the DNA after Cr treatment to the amount of amino acid released from control DNA was determined.

Figure 5. Concentration dependence in the association of amino acids with DNA. CHO cells were treated with 100 μM potassium chromate for 4, 8, 16, and 20 hr in complete media. Samples of amino acids were prepared as described in Materials and Methods.

Discussion

Radioisotopically labeled threonine was associated with DNA following chromate treatment. An increase in the presence of threonine compared to controls was observed. The results suggest that free amino acids react readily with DNA in intact cells to produce DNA-amino acid complexes. Additionally, we used a recently developed nonprotease method to isolate cellular DNA following chromate treatment. Protein–DNA complexes were separated by precipitation with K+-SDS of DNA mechanically fragmented to homogeneous sizes while DNA not precipitated by the K+-SDS had little or no DNA–protein cross-links. Examination of this soluble DNA revealed substantial increases in the amount of amino acid DNA complexes in chromate-treated cells compared to controls (data not shown).

Figure 6 shows time-dependence in the complexing of amino acids with DNA. Relatively long incubation periods were required to produce substantial increases in amino acid DNA complexes (16–20 hr).

To evaluate whether the extraction procedure measures amino acid residues from protein–DNA cross-links or cross-linking of free amino acids with DNA, we reacted DNA with BSA in the absence or presence of trivalent Cr(III) under conditions in which we have shown that BSA could be cross-linked to the DNA Cr (10). We also reacted DNA with amino acids in the absence and presence of trivalent Cr. Figure 7 illustrates that when BSA was cross-linked to the DNA by Cr, there was little increase of amino acids above background. However, when free amino acids were cross-linked with DNA by Cr(III), there was a substantial increase of amino acids complexed with DNA above the background. When protein synthesis was inhibited by cycloheximide, more radioisotopically labeled threonine was associated with DNA following chromate treatment than when it was not inhibited (Figure 8). These results suggest that free amino acids react readily with DNA in intact cells to produce DNA–amino acid complexes. Additionally, we used a recently developed nonprotease method to isolate cellular DNA following chromate treatment (6). Protein–DNA complexes were separated by precipitation with K+-SDS of DNA mechanically fragmented to homogeneous sizes while DNA not precipitated by the K+-SDS had little or no DNA–protein cross-links. Examination of this soluble DNA revealed substantial increases in the amount of amino acid DNA complexes in chromate-treated cells compared to controls (data not shown).

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these amino acids were not found to be increased when radiolabeled amino acids were used. The reason for this is presently unknown but may have to do with radiolabeling problems. Most of the cysteine associated with DNA using radiolabeled amino acids was found to originate from glutathione cross-links induced by chromate (11). This was demonstrated by inhibiting protein synthesis with cycloheximide, which actually stimulated the amount of cysteine associated with DNA (11). Thus, the cysteine–DNA cross-links do not represent residual proteins associated with the DNA, but most probably represent actual glutathione–DNA adducts. In fact, most cysteine in proteins is generally not accessible for cross-linking in intact cells.

The fact that glutamine and glutamic acid were found to be highly cross-linked to the DNA in the present study when a nonradioactive method was used may be due to the high levels of glutamine that were added in the tissue-culture media. In fact, using radioactive glutamine, the specific activity of glutamine would be so low that glutamine DNA cross-linking may not be detected by these radioactive methods. These results suggest that, in fact, the residual amino acids associated with DNA did not, in large part, originate from protein cross-linked with DNA, but originated from free amino acid pools or small peptides that were cross-linked to the DNA in intact cells. This was certainly the case with glutathione that was found complexed with the DNA (11). Most of the cysteine that was found complexed with the DNA originated from glutathione (11). Experiments conducted here also suggest that when albumin was cross-linked to the DNA, there was little residual amino acid complexed with DNA; however, when free amino acids were cross-linked to DNA and the DNA was isolated by standard methods, there was an abundance of amino acid. Further, when protein synthesis was inhibited in intact cells, there was more radiolabeled amino acid cross-linked to the DNA, suggesting that free amino acids do, in fact, react with the DNA. Although there may be some contribution from residual protein–DNA cross-links, a substantial percentage of the amino acids associated with DNA probably originated from the reaction of free amino acids with DNA. In general, free amino acids are chemically more reactive than amino acids complexed as peptides.

We previously studied the chemistry of the cross-linking of amino acids to the DNA and demonstrated in the case of chromate that trivalent Cr was involved in the cross-link, since DNA-bound cysteine could be extracted with EDTA (11). Trivalent Cr is the only form of chromate that can be chelated by EDTA, and we are not studying amino acid cross-links except those involving trivalent Cr with EDTA. However, we have also observed in previous studies that amino acids can be cross-linked to the DNA by oxidative mechanisms that do not actually involve trivalent Cr (11).

The biologic significance of the amino acids cross-linked to the DNA is not currently understood; however, these were thought to be amino acid residues arising from chromatin-scaffold proteins (1–3). We show here that peptides and free amino acid cross-linking in intact cells must also be considered as a possible major origin of these amino acid cross-links. The fact that one can demonstrate such a large increase in amino acids associated with DNA following treatment with cross-linking agents indicates that these amino acid–DNA complexes may be a substantial impediment to DNA replication that could possibly lead to mutations and may be responsible for carcinogenic effects of chromate. Complexes of DNA and amino acids probably enhance the stability of the Cr bound to the DNA. An additional utilization of this lesion as a potential biomarker of human exposure to cross-linking agents is possible since amino acids associated with DNA can be quantitated with high precision.
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