Guanine and 7,8-Dihydro-8-Oxo-Guanine–Specific Oxidation in DNA by Chromium(V)

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The hexavalent oxidation state of chromium [Cr(VI)] is a well-established human carcinogen, although the mechanism of cancer induction is currently unknown. Intracellular reduction of Cr(VI) forms Cr(V), which is thought to play a fundamental role in the mechanism of DNA damage by this carcinogen. Two separate pathways of DNA damage, an oxidative pathway and a metal-binding pathway, have been proposed to account for the lesions observed in cell systems. We have used a model Cr(V) complex, N,N'-ethylenebis(salicylidene-animato)oxochromium(V) [Cr(V)-Salen], to investigate the oxidative pathway of DNA damage and to elucidate the lesions generated from this oxidation process. Reaction of Cr(V)-Salen with synthetic oligonucleotides produced guanine-specific lesions that were not 8-oxo-2′-deoxyguanosine, based on the inability of iridium(IV) to further oxidize these sites. Oxidation products were identified using a 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-G) containing oligonucleotide to increase the yields of product for identification by electrospray ionization mass spectrometry. The guanine-based lesions observed by mass spectrometry corresponded to the lesions guanidinohydantoin and spiroiminodihydantoin. The effects of these Cr(V)-Salen–induced lesions on DNA replication fidelity was assayed using a polymerase-based misincorporation assay. These lesions produced G→T transversion mutations and polymerase stops at levels greater than those observed for 8-oxo-G. These data suggest a model by which chromate can cause DNA damage leading to mutations and cancer. *Key words:* chromate, chromium(V), 7,8-dihydro-8-oxo-2′-deoxyguanine, guanidinohydantoin, oxidative damage, spiroiminodihydantoin. *Environ Health Perspect* 110(suppl 5):725–728 (2002). http://ehpnet1.niehs.nih.gov/docs/2002/suppl-5/725-728sudgen/abstract.html

Exposure of cellular systems to the human carcinogen chromate [Cr(VI)] results in a wide variety of DNA lesions. Some of the lesions formed from chromate treatment are strand breaks, nucleic acid base modifications, DNA interstrand and intrastrand cross-links, and DNA–protein cross-links (*1–4*). Although these lesions have been demonstrated in a variety of cellular and noncellular systems, little is known about the fundamental mechanism of interaction between chromate and DNA that give rise to the specific lesions formed from this reaction. With few exceptions (*5*), biomarkers corresponding to specific lesions derived from chromate exposure have not been adequately described.

Chromate is unidirectionally accumulated into cells by active transport through anion channels on the basis of its structural similarity to sulfate and phosphate (*6*). Once internalized, chromate is reduced by endogenous cellular reductants to form a variety of potential DNA-damaging species, including the highly reactive Cr(V) oxidation state of the metal and oxygen-, carbon-, or sulfur-centered radical species (*7*). The final stable state of chromium intracellularly is Cr(III), and this oxidation state may also play a role in the DNA damage associated with the metal.

A confounding factor in the determination of a mechanism(s) of DNA damage by chromate is the large number of potential cellular reductants, the associated myriad oxidizing species formed during the reduction process, and the variety of lesions observed upon cellular treatment with this metal.

The endogenous reductant responsible for activation of chromate toward DNA damage in cellular systems continues to be a contentious issue. Various cellular reductants such as glutathione, ascorbate, NADPH and cysteine have all been observed to reduce chromate *in vitro* and *in vivo* (*8–10*). These reduction pathways form the highly reactive Cr(V) oxidation state, although many also form radical species. It is the confounding co-generation of radical species that has led to the different mechanistic descriptions for DNA damage by chromate. However, it has recently been shown that many types of DNA damage and markers of oxidative stress can also be formed through a direct oxidation mechanism involving transient high-valent oxidation states of chromium such as Cr(V) (*11,12*).

A broad mechanistic description of DNA damage from chromate exposure has postulated a bifurcated pathway whereby various DNA-damaging species result from either an oxidative pathway or a metal-binding pathway. The oxidative pathway would account for the frank strand breaks, abasic sites, and base modifications observed with this carcinogen, whereas the metal-binding pathway would account for the interstrand and intrastrand cross-linking and the DNA–protein cross-linking observed for chromate.

Our interest has focused primarily on the oxidative pathway of DNA damage by chromate. Cellular data have implied that this pathway is a significant contributor to the overall mutagenic and carcinogenic potential of this metal (*13*). Specifically, the reaction of chromium with the nucleic acid base guanine is of interest because of the number of studies that have indicated a preference for high-valent chromium to react at this site (*14–16*).

In this study, we examined the base-specificity of oxidation of DNA when reacted with a model high-valent chromium(V) complex and have identified candidate lesions formed from this reaction. A profound specificity of oxidation toward guanine residues within the DNA strand was observed. The guanine-based lesions of guanidinohydantoin (GH) and spiroiminodihydantoin (SH) were identified when the reactions were carried out using a 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-G)–containing oligonucleotide. The impact that these modified guanine lesions have on mutations was determined using a polymerase stop assay. Significant levels of G→T transversions and polymerase stops were observed.

Materials and Methods

Cr(V) Synthesis

N,N'-ethylenebis(salicylidene-animato) oxochromium(V), or Cr(V)-Salen was synthesized in the trivalent oxidation state as the hexafluorophosphate salt, followed by oxidation to the pentavalent form with iodosylbenzene (*17*). The structure was confirmed by UV-visible spectroscopy and electron spin resonance spectroscopy.

Cr(V) Reactions with DNA

Unmodified oligonucleotides used for this study were synthesized using standard automated solid-state methods. The 8-oxo-G containing oligonucleotide was synthesized by Trilink Biotechnology Inc. (San Diego, CA, USA). Oligonucleotides used in these studies were based on the 25-mer oligo sequence

5′-d[ATGGCGTAATCAT**X**GTCATAGCT GT]-3′, where **X** at position G_{14} is either 8-oxo-G or the unmodified G base. Purification of the oligonucleotides prior to reaction and after oxidation with Cr(V)-Salen was accomplished by high-performance liquid chromatography (HPLC) using a Dionex Nucleopac PA-100 4 × 250-mm anion-exchange column (Dionex Corp., Sunnyvale, CA, USA).

Reactions between 50–250 µM Cr(V)-Salen and 10–100 µM oligonucleotide were carried out in 10 mM sodium phosphate buffer (pH 6.0–7.0) in 100-µL volumes. Reactions were allowed to proceed at room temperature for 30 min prior to analysis. Residual chromium was removed from the reaction mixtures using a Nensorb-20 DNA purification cartridge (NEN Life Science Products Inc., Boston, MA, USA). The DNA-containing fraction was evaporated to dryness and loaded on a 20%, 0.4-mm thickness, 21 cm × 50 cm denaturing (7 M urea) polyacrylamide gel with 4 µL 80% formamide loading buffer containing 0.05% xylene cyanol and bromophenol blue. Alkaline-labile cleavage sites on the DNA were analyzed by treating lyophilized samples of the reaction mixture with 100 µL of a 1.0 M solution of freshly prepared piperidine followed by heating at 90°C for 30 min. Electrophoresis was carried out at 2,200 V and 24 milliamps, with 1× Tris:boric acid:EDTA as the running buffer. Visualization of the DNA cleavage products was performed using autoradiography.

DNA Oxidation Product Analysis by Electrospray Ionization Mass Spectrometry

DNA oxidation products from the reaction with Cr(V)-Salen were purified by HPLC, resuspended in an aqueous buffer containing 2.5 mM imidazole and 2.5 mM piperidine, and analyzed by electrospray ionization mass spectrometry (ESI–MS). ESI–MS spectra were obtained on a Micromass Quattro II tandem mass spectrometer (Micromass UK Ltd., Manchester, UK). The oligomers were introduced into a QTOF (quadrapole time of flight) mass spectrometer by direct infusion via a syringe pump at a flow rate of 5 µL/min. The capillary voltage was set to –2,200 volts, and ion signals were detected in the negative ion mode. The initial spectra were charge-state deconvoluted using the transform algorithm featured in the Micromass MassLynx v. 3.4 software package (Micromass).

Primer Extension Mutation Assay

Modified oligo (template) with sequence 5′-d[TCATGGGTC**X**TCGGTATATCAG TGCTATCACATTAGTGTA]-3′ containing an 8-oxo-G at position **X** was reacted with 50 µM Cr(V)-Salen as described above. The primer extension assay was run directly after removal of chromium through a NENSORB purification cartridge, or the products of the oxidation were separated and purified by HPLC as discussed above. The modified oligonucleotide was lyophilized to dryness and redissolved in 10 µL 10.0 mM Tris-HCl (pH 7.5) containing 5.0 mM $MgCl_2$ and 7.5 mM dithiothreitol. A 5′-^{32}P-labeled primer with the template complementary sequence of 5′-d[TGATAGCACTGATATACCGA]-3′ was added at a template/primer ratio of 9:1 and annealed by heating to 90°C for 5 min, followed by slow cooling to room temperature over 2.5 hr. DNA extension was initiated by the addition of 0.1 U of exo⁻ Klenow fragment and either 100 µM of the individual deoxynucleoside triphosphates (dNTP) or 100 µM of a mixture of all four dNTPs. The reaction was incubated for 20 min at 37°C prior to reaction termination by addition of 10 µL loading buffer, and electrophoretic analysis was carried out on a denaturing 15% polyacrylamide gel. Visualization was by autoradiography as described above.

Results

Reaction of Cr(V) with Unmodified Oligonucleotides

Reaction of the model Cr(V) complex Cr(V)-Salen with a synthetic oligonucleotide resulted in oxidation at each guanine residue within the strand to yield piperidine-labile strand breaks (Figure 1; lane 4). Neither the Cr(III)-Salen nor the oxidant iodosylbenzene showed appreciable strand cleavage under these conditions (Figure 1; lanes 2, 3). Posttreatment of the Cr(V)-Salen–oxidized oligonucleotide with Ir(IV) (Figure 1; lane 6), an 8-oxo-G–specific oxidant, did not show an increase in the level of strand breaks, suggesting that base modification induced by Cr(V)-Salen was not the 8-oxo-G moiety. 8-oxo-G is poorly piperidine labile (*18*) but is thermodynamically labile toward further oxidation (*19*). The oxidation potential of guanine is 1.29 V versus the normal hydrogen electrode, whereas that of the 8-oxo-G–modified base has an oxidation potential of 0.64 V (*19*). The failure of Ir(IV) posttreatment to increase piperidine lability suggested that any 8-oxo-G formed during the reaction had been further oxidized to a fully piperidine-labile lesion. The use of unmodified DNA to observe base lesions derived from Cr(V)-Salen treatment generated relatively low levels of oxidized guanine products and lacked site specificity. These two drawbacks made the system refractory to conventional analytical methods for elucidation of the oxidized lesions formed in this system. We have circumvented these problems of low reactivity and site specificity by reacting the

Cr(V)-Salen complex with an oligonucleotide incorporating the oxidatively labile 8-oxo-G group, the putative intermediate in the formation of further oxidized guanine lesions.

Reaction of Cr(V) with 8-Oxo-G–Modified Oligonucleotides

Reaction of Cr(V)-Salen was carried out with a modified oligonucleotide identical to that used in Figure 1, with the exception that the guanine at position 14 was substituted with an 8-oxo-G. The reaction was carried out with 50–250 µM Cr(V)-Salen and yielded base-specific oxidation at the site of modification on the DNA (Figure 2; lanes 2–4). This was in contrast to the unmodified strand, where oxidation at each guanine residue was observed. The 8-oxo-G–specific oxidant Ir(IV) showed the expected base-specific reaction (Figure 2; lane 5). The Cr(III)-Salen and iodosylbenzene controls showed no increased oxidation of the 8-oxo-G–containing oligonucleotide (Figure 2; lanes 7,8). Significantly higher levels of oxidation were observed for the modified oligonucleotide (8-oxo-G containing) versus the unmodified oligonucleotide. The high product yield and site specificity obtained with the modified oligonucleotide was necessary to allow lesion identification and determination
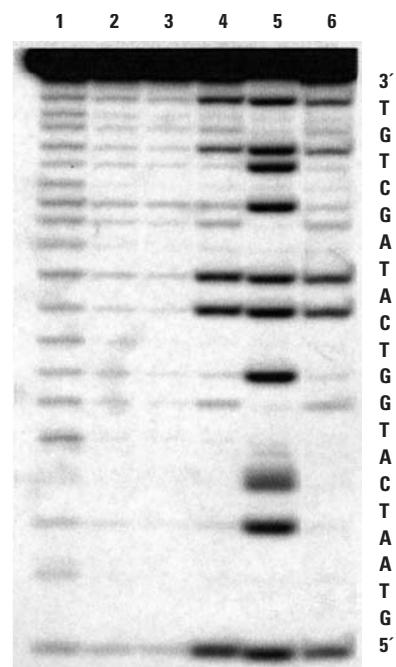

Figure 1. Autoradiogram of the piperidine-treated 25 base-pair oligonucleotide 5′-d[ATGGCGTAAT-CATGGTCATAGCTGT]-3′ showing guanine-specific oxidation with Cr(V)-Salen treatment. Lane 1: 10 µM DNA; lane 2: 10 µM DNA + 100 µM Cr(III)-Salen; lane 3: 10 µM DNA + 100 µM iodosylbenzene; lane 4: 10 µM DNA + 100 µM Cr(V)-Salen; lane 5: Maxam-Gilbert guanine/adenosine (G/A) lane; lane 6: 10 µM DNA + 250 µM Na_2IrCl_6. Reproduced with permission from Sugden et al. (*12*) with permission of the American Chemical Society.

of mutations using an *in vitro* polymerase misincorporation assay.

Identification of Oxidized Lesions in DNA Derived from Cr(V)-Salen Treatment

The reaction products derived from Cr(V)-Salen oxidation of the 8-oxo-G–containing oligo were separated using anion exchange HPLC (data not shown). Peaks of four major products were observed, and each peak was purified and analyzed by ESI–MS (Figure 3). Peak 3 co-eluted on the HPLC with unreacted oligo and gave an identical mass to charge ratio (m/z = 7,727 amu) as that of the

control oligo. Peaks 1 and 2 gave identical M – 10 mass shifts from the unreacted oligo, with m/z = 7,717 amu. This 10-amu mass shift for peaks 1 and 2 has been identified as GH and its isomer iminoallantoin (IA) (*20*). These two isomers readily interconvert, and at this time it is not possible to determine which isomer corresponds to which peak. Peak 4 yielded a M + 16 mass shift (m/z = 7,743 amu) from that of the unreacted oligo and has been identified as the further oxidized guanine base-product SH (*20*). All product assignments were based on previous reactions with the 8-oxo-G nucleoside (data not shown), characteristic mass shifts, and literature precedent.

Polymerase Arrest and Base Misincorporation opposite Oxidized Lesions

Mutational analysis of Cr(V)-Salen–treated oligonucleotides was carried out using a primer extension assay and the exo⁻ Klenow
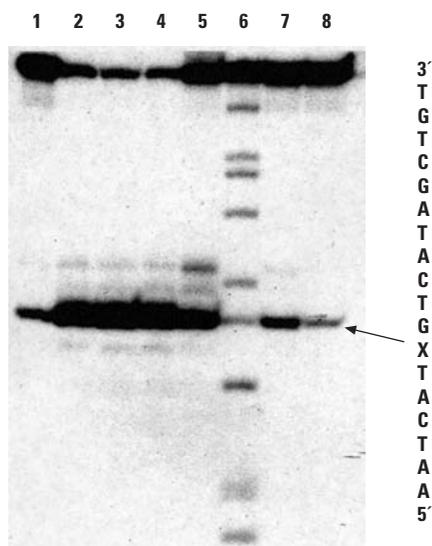

Figure 2. Autoradiogram of the piperidine-treated, 25 base-pair 8-oxo-G, X, modified oligonucleotide 5′-d[ATGGCGTAATCATXGTCATAGCTGT]-3′ showing specific oxidation at G_{14} after Cr(V)-Salen treatment. Lane 1: 10 μM DNA; lane 2: 10 μM DNA + 50 μM Cr(V)-Salen; lane 3: 10 μM DNA + 100 μM Cr(V)-Salen; lane 4: 10 μM DNA + 250 μM Cr(V)-Salen; lane 5: 10 μM DNA + 250 μM Na_2IrCl_6; lane 6: Maxam-Gilbert G/A lane; lane 7: 10 μM DNA + 100 μM iodosylbenzene; lane 8: 10 μM DNA + 100 μM Cr(III)-Salen.
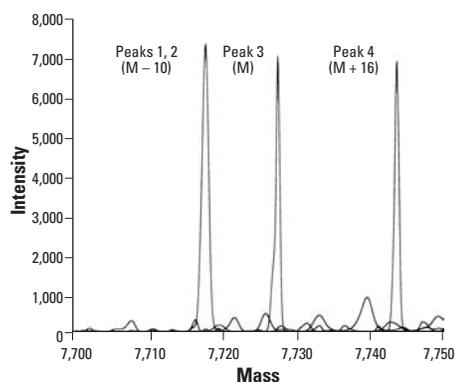

Figure 3. ESI–MS spectra of products formed from the reaction of the 8-oxo-G–containing oligo with Cr(V)-Salen.
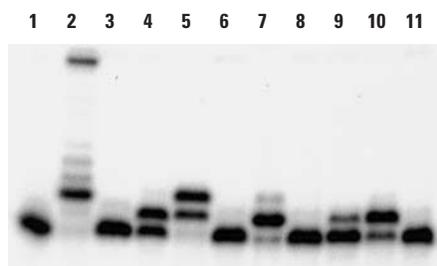

Figure 4. Autoradiogram of the primer extension assay carried out using the exo⁻ Klenow fragment on 8-oxo-G–modified DNA (lanes 1–6) and Cr(V)-Salen–treated 8-oxo-G–modified DNA (lanes 7–11). Lane 1: primer control; lane 2: DNA template with all four deoxynucleoside triphosphates; lane 3: DNA template + deoxyguanosine triphosphate; lane 4: DNA template + deoxycytidine triphosphate; lane 5: DNA template + deoxyadenosine triphosphate; lane 6: DNA template + thymidine triphosphate; lane 7: DNA template + deoxynucleoside triphosphate mix; lane 8: DNA template deoxyguanosine triphosphate; lane 9: DNA template + deoxycytidine triphosphate; lane 10: DNA template + deoxyadenosine triphosphate; lane 11: DNA template + thymidine triphosphate. Reproduced from Sugden et al. (*12*), with permission of the American Chemical Society.
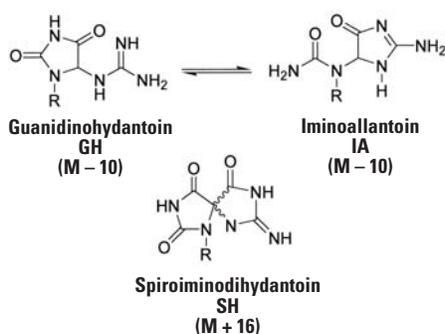

Figure 5. Structures of the oxidized guanine lesions.

fragment polymerase. Primer extension using the 8-oxo-G–modified template and all four dNTPs without Cr(V)-Salen treatment partially arrested the DNA polymerase, although a significant amount of full-length template was formed (Figure 4; lane 2). When the primer extension assay was carried out in the presence of only one of the dNTPs, misincorporation of adenine occurred as well as incorporation of the correct base cytosine (Figure 4; lanes 4, 5). After Cr(V)-Salen treatment of the template, complete polymerase arrest was observed with all four dNTPs present (Figure 4; lane 7). A relative increase in the misincorporation of adenine over cytosine was also observed (Figure 4; lanes 9, 10). When the oxidized species detected by ESI–MS shown in Figure 3 were purified by HPLC and assayed for base misincorporation, a nearly complete misincorporation of the base adenine was observed opposite the SH and GH/IA lesions (data not shown).

Discussion

Until recently, the primary lesion thought to result from oxidation of guanine was 8-oxo-G. It is becoming clear that the oxidation properties that make 8-oxo-G amenable to observation by electrochemical detection also make this species prone to degradation into further oxidized products. A variety of further oxidized species of 8-oxo-G have been observed from treatment with different oxidizing agents. These include oxazolone, imidazolone, and cyanuric acid (*21,22*), as well as the oxidized species observed in this study [guanidinohydantoin/iminoallantoin and spiroiminodihydantoin (*20*)] (Figure 5).

With few exceptions, exposure of cellular systems to Cr(VI) have failed to show formation of the classical biomarker of oxidative damage, 8-oxo-G. These data show that a better biomarker for base oxidation induced by Cr(VI) may be these further oxidized guanine products. At present, no reliable cellular assay for these products exist to test this hypothesis.

Predominant mutations observed in a variety of different cellular systems after Cr(VI) exposure have been G→T transversions (*23,24*). These data show that the G→T transversion mutations may be accounted for by the formation of these further oxidized guanine lesions. It should be noted, however, that other lesions form G→T transversion mutations, including 8-oxo-G demonstrated in this study and a variety of bulky adducts. The induction of polymerase arrest upon cellular treatment with Cr(VI) was demonstrated previously (*15*). Polymerase arrest in this system has normally been attributed to adducted chromium. These data show that induction of polymerase arrest may also be attributable to the formation of these further oxidized guanine lesions.

The use of Cr(V)-Salen as a model for the activated form of chromium upon cellular internalization has demonstrated many of the hallmarks of oxidative DNA damage attributed to Cr(VI) exposure in cells. These results are significant in that they show the first guanine-specific lesions generated by the direct interaction of chromium with DNA without addition of exogenous oxidant. We believe these studies may help to *a*) define the mechanisms of DNA damage that lead to cancer upon Cr(VI) exposure, and *b*) reveal novel biomarkers to assess Cr(VI) exposure in cellular systems.

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