Induction of DNA damage by carcinogetic hexavalent chromium compounds \([\text{Cr(VI)}]\) results from its reduction to lower oxidation states. Reductive metabolism of \(\text{Cr(VI)}\) generates intermediate \(\text{Cr(III)}\)-related species, organic radicals, and finally \(\text{Cr(III)}\), which forms stable complexes with many biological ligands, including DNA. To determine the biological significance of different reaction products, we examined genotoxic responses and the formation of DNA damage during reduction of \(\text{Cr(VI)}\) by its biological reducer, cysteine. We have found that cysteine-dependent activation of \(\text{Cr(VI)}\) led to the formation of \(\text{Cr–DNA}\) and cysteine–\(\text{Cr–DNA}\) adducts as well as interstrand DNA cross-links. The yield of binary and ternary DNA adducts was relatively constant at different concentrations of \(\text{Cr(VI)}\) and averaged approximately 54 and 45%, respectively. Interstrand DNA cross-links accounted on average for 1% of adducts, and their yield was even less significant at low \(\text{Cr(VI)}\) concentrations. Reduction of \(\text{Cr(VI)}\) in several commonly used buffers did not induce detectable damage to the sugar–phosphate backbone of DNA. Replication of \(\text{Cr(VI)}\)-modified plasmids in intact human fibroblasts has shown that cysteine-dependent metabolism of \(\text{Cr(VI)}\) resulted in the formation of mutagenic and replication-blocking DNA lesions. Selective elimination of \(\text{Cr–DNA}\) adducts from \(\text{Cr(VI)}\)-treated plasmids abolished all genotoxic responses, indicating that nonoxidative, \(\text{Cr(III)}\)-dependent reactions were responsible for the induction of both mutagenicity and replication blockage by \(\text{Cr(VI)}\). The demonstration of the mutagenic potential of \(\text{Cr–DNA}\) adducts suggests that these lesions can be explored in the development of specific and mechanistically important biomarkers of exposure to toxic forms of \(\text{Cr}\).

Hexavalent chromium compounds \([\text{Cr(VI)}]\) are recognized human and animal carcinoogens \((1,2)\). The cell transformation ability of \(\text{Cr(VI)}\) is believed to result from its mutagenic, clastogenic, and other genotoxic activities \((3,4)\). \(\text{Cr(VI)}\) itself is unreactive toward DNA at physiological pH and requires reductive activation to produce DNA-damaging species. The most important intracellular reducers of \(\text{Cr(VI)}\) are ascorbate and cysteine. \(\text{Cr(VI)}\) has been shown to be reduced by these biological reductants to \(\text{Cr(III)}\) (5–7). The reactive metabolites of \(\text{Cr(VI)}\) generate intermediate \(\text{Cr(V)}\) and \(\text{Cr(IV)}\) forms and finally yields thermally stable \(\text{Cr(III)}\) \((8,9)\). The relative amount of these intermediate \(\text{Cr}^{x}\) forms varies dramatically depending on the experimental conditions, such as the nature of the reducing agent and its ratio to \(\text{Cr(VI)}\). Other products of \(\text{Cr(V)}\) reduction include sulfur- and carbon-based radicals \((9–11)\). Hydrogen peroxide, either added intentionally or generated incidentally from iron-contaminated reagents, is responsible for the formation of hydroxyl radicals and \(\text{Cr(V)}\)-peroxo complexes during reduction of \(\text{Cr(VI)}\) \((10,12)\). These unstable \(\text{Cr}\) intermediates and radical species can potentially induce oxidation of DNA \((13)\), which could subsequently lead to mutations and chromosomal damage. DNA-damaging activity of hydroxyl radicals is well known, whereas the reactivity of thiol and carbon-based radicals is expected to be much lower \((11,14)\). Treatment of cells or reduction of \(\text{Cr(VI)}\) \(\text{in vitro}\) also generates numerous \(\text{Cr–DNA}\) adducts. These include \(\text{Cr(III)}\)-DNA adducts, \(\text{Cr(III)}\)-mediated DNA cross-links with \(\text{Cys}\), histidine, or thiolatone \((15)\), as well as interstrand DNA cross-links \((16)\). \(\text{Cr(III)}\)-DNA adducts have been found to be mutagenic during replication in human fibroblasts \((17)\).

The presence of several transient species and the potential formation of both oxidative and \(\text{Cr–DNA}\) lesions raise two important questions: \(a\) what is the nature of DNA-attacking species, and \(b\) what is the relative significance of oxidative and \(\text{Cr(III)}\)-dependent pathways in the induction of genotoxicity by \(\text{Cr(VI)}\)? To address these questions, we have conducted detailed studies of \(\text{Cys}\)-based reactions in which direct determinations of DNA damage and the assessment of genotoxic consequences were performed \((18–20)\). Here, we summarize our findings that nonoxidative mechanisms involving \(\text{Cr(III)}\)-DNA binding are responsible for the induction of the genotoxicity during reductive activation of \(\text{Cr(VI)}\) by \(\text{Cys}\).

**Experimental Procedures**

All buffers and reagents for electrophoresis were from Sigma (St. Louis, MO, USA). Chelex-100 resin and Bio-Gel P-30 columns were obtained from Bio-Rad (Hercules, CA, USA). \(\text{K}_2\text{CrO}_4\) (A.C.S. reagent) was from Aldrich (Milwaukee, WI, USA), \(2’,7’\)-dichlorofluoroscin diacetate was supplied by Molecular Probes (Eugene, OR, USA), \(\text{Na}_2\text{Cr}_2\text{O}_7\), and \(\text{[}^{35}\text{S}\text{]}\)-labeled \(\text{t-Cys}\) were from Amersham (Arlington Heights, IL, USA). \(\Phi\text{X}\text{174}\) DNA was obtained from New England Biolabs (Beverly, MA, USA). \(\text{t-Cysteine}\) was from Gibco (Rockville, MD, USA). All reagents were purified by Chelex-100 chromatography as described previously \((18)\). Solutions of \(\text{Cys}\) were used within 30 min of preparation. A standard \(\text{Cr(VI)}\) reduction mixture contained 2 µg of supercoiled pSP189 DNA, 25 mM 3-[N-morpholino]-propanesulfonic acid (MOPS; pH 7.0), 2 mM \(\text{Cys}\), and various concentrations of \(\text{K}_2\text{CrO}_4\) in a final volume of 50 µL. In some reactions 5 mM ethylenediamine tetraacetic acid (EDTA) was also added or MOPS buffer was replaced by 25 mM phosphate, 2-[N-morpholino]ethanesulfonic acid (MES) or HEPES (pH 7.0). Samples used for the detection of DNA breakage contained 0.3 µg of supercoiled \(\Phi\text{X}174\) DNA in a final volume of 25 µL. All samples were incubated for 60 min at 37°C, and DNA-unbound reactants were removed by Bio-Gel P30 columns and precipitation with ethanol. The amounts of DNA-bound \(\text{Cr}\) and \(\text{Cys}\) were determined using trace quantities of radioactive \(\text{[}^{51}\text{Cr}\text{]}\)-chromate and \(\text{[}^{35}\text{S}\text{]}\)-Cys, respectively. The number of interstrand DNA cross-links was calculated from the relative amounts of double-stranded DNA after denaturation in 0.2 M NaOH and separation of DNA by agarose electrophoresis \((18)\). Dissociation of DNA-bound \(\text{Cr}\) was achieved by incubating adducted DNA in the presence of 50 mM sodium phosphate (pH 7.0) for 24 hr at 37°C \((19)\). Released \(\text{Cr}\) was removed by Bio-Gel P30 columns. The presence of nicked DNA was analyzed by agarose electrophoresis.

The genotoxic significance of DNA damage arising from \(\text{Cr(VI)}\) reduction by \(\text{Cys}\) was examined using the pSP189 shuttle-vector DNA replication assay. This article is part of the monograph *Molecular Mechanisms of Metal Toxicity and Carcinogenicity*, Address correspondence to A. Zhitkovich, Dept. of Pathology and Laboratory Medicine, Brown University, Box G-B511, Providence, RI 02912 USA. Telephone: (401) 863-2912. Fax: (401) 863-9008. E-mail: Anatoly.Zhitkovich@brown.edu

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The formation of Cr(III)–DNA adducts (19). This vector is capable of replicating in intact human cells, which permits its use for the biological assessment of the presence of mutagenic and replication-blocking DNA lesions after in vitro treatments with Cr(VI). In brief, control and Cr(VI)-treated plasmids were transfected into human fibroblasts and were allowed to replicate for 42–48 hr. Replicated plasmids was isolated and then used to transfect an indicator Escherichia coli strain, MBL50. The efficiency of plasmid replication in human cells was determined by the number of E. coli transformants (ampicillin-resistant colonies). The mutation frequency at the supF gene was calculated by dividing the number of arabinose/ampicillin-resistant colonies by the number of ampicillin-resistant colonies.

**Results and Discussion**

Kinetic studies of Cys-dependent reduction of Cr(VI) at neutral pH have indicated that the initial step in the reduction could proceed through either one- or two-electron transfer (20). The relative contribution of each reduction pathway depends on the concentration of Cys, with one-electron transfer dominating in the physiological range of Cys concentrations (>90% reduction). Transfer of one electron to Cr(VI) would generate a sulfur-based thyl radical and Cr(V) as the first products. Increased oxidation of 2’,7’-dichlorofluoroscin in the reactions containing low Cys concentrations was consistent with the enhanced production of Cr(V) under these conditions (20). The formation of Cr(V) species and thyl radicals in Cr(VI)/Cys mixtures has been detected using electron spin resonance (8,10). A second reduction step most likely involves the production of Cr(IV), but this has not yet been established experimentally. Cr(III)–(Cys)$_2$ complex was the major stable product of Cr(VI) reduction by Cys (21). Addition of DNA to the reduction mixtures resulted in the formation of Cr(III)–DNA adducts (18). Therefore, the induction of genotoxicity in Cys-based reactions can potentially result from oxidative damage by Cr(V) and thyl radicals or/and is caused by Cr(III)–DNA binding.

The sugar–phosphate backbone of DNA is one of the most common sites of damage by oxidizing species. To detect the presence of DNA-oxidizing activity in Cr(VI)–Cys reactions, we analyzed the production of DNA single-strand breaks (Figure 1A). No significant DNA breakage was detected using sodium phosphate or three organic buffers. Reduction of Cr(VI) in phosphate or MOPS buffer using a 10-fold range of Cys concentrations also failed to produce abasic sites (20). Thus, thyl radicals and Cr(V) species generated in Cr(VI)–Cys reactions appeared unable to cause oxidative damage to the sugar–phosphate backbone of DNA.

Reduction of Cr(VI) results in the formation of three types of Cr–DNA adducts: binary Cr–DNA, ternary Cys–Cr(III)–DNA adducts, and interstrand DNA cross-links (18). Binary and ternary adducts represented the majority of Cr–DNA complexes, and their yield was essentially constant over a 25- to 100-µM range of Cr(VI) (Figure 1B). The average yields of binary and ternary DNA adducts were 53.8 ± 1.8% and 45.3 ± 2.1%, respectively. In contrast to monoaducts, the formation of DNA–DNA cross-links was progressively higher with increasing Cr(VI) concentrations [0.2, 1.0, and 2.2% yield at 25, 50, and 100 µM Cr(VI), respectively]. Samples containing 10 µM Cr(VI) had undetectable amounts of DNA cross-links (18). Although DNA cross-links are considered to be potent polymerase-blocking lesions (22), their sparse formation at low concentrations...
of Cr(VI) suggests that these lesions are not very likely to play a major role in the genotoxicity induced by Cys-dependent activation of Cr(VI).

To determine whether reduction of Cr(VI) in the presence of Cys produced genotoxic DNA modifications, we employed a pSP189 shuttle-vector approach (19). In this assay, the pSP189 DNA is incubated in Cr(VI)/Cys reduction mixtures, purified, and then transfected into human cells to assess the presence of premutagenic and replication-blocking DNA lesions. Using this methodology, it is possible to examine the importance of different types of DNA damage by controlling their formation during in vitro reduction of Cr(VI). Figure 2A shows that the formation of Cr–Cys adducts can be completely blocked when reaction mixtures contained the Cr(III)-chelating agents EDTA or inorganic phosphate. We have also found (19) that the majority of Cr(III)–DNA adducts formed in the MOPS-based reactions can be dissociated by the subsequent incubation in the presence of 50 mM phosphate (Figure 2A, bar V). When pSP189 plasmids modified in Cr(VI)–Cys reactions were replicated in human fibroblasts, we found a strong mutagenic response in samples containing Cr–DNA adducts but not in those lacking them (Figure 2B). These results demonstrate that Cys-dependent metabolism of Cr(VI) did produce mutagenic DNA damage and that Cr–DNA adducts were responsible for the induction of mutagenicity. In addition to mutagenic effects, Cr(VI)-induced damage also caused a strong inhibition of plasmid replication intact human cells (Figure 2C, bar II). Blocking of Cr(III)–DNA binding in the reactions containing EDTA or phosphate essentially restored template properties of pSP189 plasmids. Release of approximately 80% of DNA-bound Cr by the treatment with phosphate resulted in almost normal yield of replicated plasmids, which further confirmed a critical role of Cr–DNA adducts in the replication-blocking activity of Cr(VI).

The shuttle-vector experiments showed that two very important forms of Cr(VI)/Cys-induced genotoxicity, mutagenesis and replication blockage are caused by nonoxidative mechanisms through the generation of Cr(III)–DNA adducts (Figure 3). These findings provide an example of the biologically relevant metabolic system in which Cr(III)–DNA adducts are responsible for the major genotoxic activities of Cr(VI). The absence of detectable oxidative DNA damage in Cr(VI)/Cys reactions indicates that thyl radicals and intermediate Cr(V/IV) forms are relatively unreactive toward DNA. We expect that the glutathione thyl radical produced in Cr(VI)/glutathione reactions (23) will have a similarly low reactivity. The demonstration of the mutagenic activity of Cr(III)–DNA adducts identifies a biologically important class of Cr(VI)-specific DNA lesions that can be explored in the development of useful biomarkers of exposure to toxic forms of Cr.

**REFERENCES AND NOTES**

1. IARC. Chromium, Nickel and Welding. IARC Monogr Eval Carcinog Risk Hum 48:49–256 (1990).
2. Sorohan T, Burgess DC, Hamilton L, Harrington JM. Lung cancer mortality in nickel/chromium platers, 1946–1995. Occup Environ Med 55:236–242 (1998).
3. Snow E. Metal carcinogenesis: mechanistic implications. Pharmacol Ther 53:31–65 (1992).
4. Landolph JR. Role of free radicals in metal-induced carcinogenesis. In: Metal ions in Biological Systems (Sigel A, Sigel H, eds). New York: Marcel Dekker, 1999;445–483.
5. Suzuki Y, Fukuda K. Reduction of hexavalent chromium by ascorbic acid and glutathione with special reference to the rat lung. Arch Toxicol 64:169–176 (1990).
6. Standeven AM, Wetterhahn KE. Ascorbate is the principal reductant of chromium(VI) in rat lung ultralattes and cytosols, and mediates chromium-DNA binding. Carcinogenesis 13:1319–1324 (1992).
7. Yuann JM, Liu KJ, Hamilton JW, Wetterhahn KE. In vivo effects of ascorbate and glutathione on the uptake of chromium, formation of chromium(III)-DNA binding and 8-hydroxy-2-deoxyguanosine in liver and kidney of Osteogenic Disorder Shionogi rats following treatment with chromium(VI). Carcinogenesis 20:1287–1275 (1999).
8. Kitagawa S, Seki H, Kametani F, Sakurai H. EPR study on the interaction of hexavalent chromium with glutathione or cysteine: production of pentavalent chromium and its stability. Inorg Chim Acta 152:251–255 (1988).
9. Stearns DM, Wetterhahn KE. Reaction of Cr(VII) with ascorbate produces chromium(VI), chromium(IV), and carbon-based radicals. Chem Res Toxicol 7:219–228 (1994).
10. Shi X, Dong Z, Dalal NS, Gannett PM. Chromium-mediated free radical generation from cysteine, penicillamine, hydrogen peroxide, and lipid peroxides. Biochim Biophys Acta 126:65–72 (1994).
11. Lay P, Levin A. Activation of molecular oxygen during the reactions of chromium(VI)/V/IV with biological reductants: implications for chromium-induced genotoxicities. J Am Chem Soc 120:6704–6714 (1998).
12. Zhang L, Lay P. EPR spectroscopic studies of the reactions of Cr(VI) with l-ascorbic, l-dehydroascorbic acid, and 5,6-epi-prollylidenyl-l-aspartic acid in water. Implications for chromium(VI) genotoxicity. J Am Chem Soc 112:12624–12627 (1990).
13. Sugden KD, Stearns DM. The role of chromium(VI) in the mechanism of chromate-induced oxidative DNA damage and cancer. J Environ Pathol Oncol 19:215–220 (2000).
14. Lalfer MV, Retel J. Contrasting effects of SH-compounds on oxidative DNA damage: repair and increase of damage. Mutat Res 295:1–10 (1993).
15. Zhitkovich A, Voitkun V, Costa M. Glutathione and free amino acids form stable complexes with DNA following exposure of intact mammalian cells to chromate. Carcinogenesis 16:907–913 (1995).
16. Bridgewater LC, Manning FCR, Patierno SR. Base-specific arrest of in vitro DNA replication by carcinogenic chromium compounds: a probe for strand crosslinking. Carcinogenesis 15:2421–2427 (1994).
17. Voitkun V, Zhitkovich A, Costa M. Cr(III)-mediated crosslinks of glutathione or amino acids to the DNA phosphate backbone are mutagenic in human cells. Nucleic Acids Res 26:2024–2030 (1998).
18. Zhitkovich A, Messer J, Shragor S. Reductive metabolism of Cr(VI) by cysteine leads to the formation of binary and ternary Cr-DNA adducts in the absence of oxidative DNA damage. Chem Res Toxicol 13:1114–1124 (2000).
19. Zhitkovich A, Song Y, Quevryw G, Voitkun V. Non-oxidative mechanisms are responsible for the induction of mutagenesis by reduction of Cr(VII) with cysteine: the role of ternary DNA adducts in Cr(VI)-dependent mutagenesis. Biochemistry 40:549–560 (2001).
20. Quevryw G, Goulart M, Messer G, Zhitkovich A. Reduction of Cr(VII) by cysteine: significance in human lymphocytes and formation of DNA damage in reactions with variable reduction rates. Mol Cell Biochem 227:107–118 (2001).
21. Kwong DWJ, Pennington DE. Stoichiometry, kinetics, and mechanisms of the chromium(VI) oxidation of l-cysteine. Inorg Chem 23:1048–1052 (1984).
22. O’Brien T, Xu J, Patierno SR. Effects of glutathione on chromium-induced DNA crosslinking and DNA polymerase arrest. Mol Cell Biochem 222:173–182 (2001).
23. Aiyar J, Berkovits HJ, Floyd RA, Wetterhahn KE. Reaction of chromium (VI) with hydrogen peroxide in the presence of glutathione: reactive intermediates and resulting DNA damage. Chem Res Toxicol 15:265–379 (1998).