Utilization of DNA–Protein Cross-Links as a Biomarker of Chromium Exposure

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Human exposure to carcinogenic Cr(VI) compounds is found among workers in a large number of professional groups, and it can also occur through environmental pollution. A significant number of toxic waste sites contain Cr as a major contaminant. In this paper we summarize our efforts to apply measurements of DNA–protein cross-links (DPC) as a test for biologically active doses of Cr(VI). DPC were found at elevated levels in lymphocytes in several human populations with low to medium Cr exposures. At high exposure to Cr(VI), exemplified by a group of Bulgarian chrome plates, DPC plateaud and adducts' levels were similar to those found in environmentally exposed individuals. Lymphocytic DPC correlated strongly with Cr levels in erythrocytes that are indicative of Cr(VI) exposure. DPC in lymphocytes were not confounded by such variables as smoking, age, body weight, gender, or ethnicity. A new version of the cross-link assay offers improved sensitivity and requires a small amount of biologic material. Preliminary results indicate that the ability of DPC to reach detectable levels at low levels of Cr exposure could be related to a lack of repair of these lesions in lymphoid cells. Cr(III)-mediated cross-links of DNA with peptide glutathione or single amino acids were mutagenic in human cells, with a relationship of higher molecular weight of the peptide/amino acid correlating with a more potent mutagenic response. We speculate that bulky DPC could also have a significant promutagenic effect. The current methodology does not allow specific determination of Cr-induced DPC; however, demonstrated sensitivity of DPC measurements and the assay's large sample capacity may allow this to be used as the initial screening test for the occurrence of DNA damage in Cr(VI)-exposed populations. — Environ Health Perspect 106(Suppl 4):969–974 (1998). http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-4/969-974zhitkovichabstract.html

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Introduction

Epidemiologic studies have consistently shown that human exposure to hexavalent Cr compounds (chromates) is associated with a higher incidence of respiratory cancers (1,2). Cr(VI) compounds are potent carcinogens in animals, as well as mutagens and mutagens in bacterial and mammalian cell-based mutagenicity assays (3–5). Cr(VI) readily enters all types of cells through a general anion channel, leading to a significant intracellular accumulation of this metal (6,7). Chromate is isosstructural with phosphate and sulfate anions, which is the basis for its rapid transport into cells and penetration into many tissues. Intracellularly, Cr(VI) undergoes reductive metabolism, ultimately forming Cr(III) (8,9). Because of extremely tight binding of Cr(III) by intracellular macromolecules, a significant portion of Cr(III) becomes trapped inside the cell for long periods of time (10). Several radical species, as well as Cr(V) and Cr(IV), have been detected during the reduction of Cr(VI) in vitro (11–14). Although numerous cellular constituents were capable of reducing Cr(VI) in vitro, in vivo reduction of Cr(VI) is believed to be driven by ascorbate and nonprotein thiols such as glutathione and cysteine (8,15,16). Formation of Cr(III) in cells leads to the induction of stable ternary DNA adducts that include proteins, glutathione, or selected amino acids cross-linked to DNA by Cr(III) (17–19).

According to recent estimates, more than 300,000 U.S. workers are potentially exposed to Cr and Cr-containing compounds in the workplace (20). Workers among approximately 80 professional groups can experience Cr exposure. The highest levels of occupational exposure to Cr and its compounds are typically found among stainless-steel welders, chrome plates, and chromate-production workers (1,2,20). Environmental contamination with Cr is also widespread and has a potential to affect large groups of the general population. Major anthropogenic sources of atmospheric Cr contamination in the United States are oil and coal combustion, steel production, chemical manufacture, primary metal production, chrome plating, and cooling towers (20). Cr release by the electroplating, leather tanning, and textile industries is largely responsible for water contamination. Disposal of Cr-containing waste products and coal ash from electrical utilities and other industries are the major sources of Cr release into the soil (1,20). Cr-containing slag produced during chromate manufacturing processes was sometimes improperly disposed of in landfill sites. For example, there have been reports indicating human exposure to Cr in Hudson County, New Jersey, where millions of tons of Cr-containing mine tailings have been used as landfill and in commercial and residential construction (21–23). Cr is a major contaminant at more than 40% of the toxic sites designated as Superfund sites by the U.S. Environmental Protection Agency (20). This number may in fact be even greater because not all Superfund sites were analyzed for possible Cr contamination.

Current biomonitoring approaches for assessing occupational exposure to Cr compounds are typically based on measurements of Cr in urine or serum. Elevated levels of Cr in these biologic fluids are well documented in workers employed in industries with high levels of exposure (24–26). Although urinary Cr screening can sometimes reveal environmental exposures (21), these measurements can be significantly

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Abbreviations used: DPC, DNA–protein cross-links; EBV-BL cells, Burkitt lymphoma cells transformed with Epstein–Barr virus; SDS, sodium dodecyl sulfate; SE, standard error.
influenced by dietary factors, which are difficult to account for. A contribution of diert-
originated nontoxic Cr(III) to serum and urine Cr levels can be significant (27,28),
leading to difficulties in assessing low-level exposures to toxic Cr(VI). Dietary Cr is
also likely to lead to high temporal variability in Cr measurements. Based on the fact
that soluble inorganic Cr(III) compounds enter cells poorly, monitoring of Cr levels in
eythrocytes was suggested to preferen-
tially assess toxic Cr(VI) exposures (10).
However, Cr(III) compounds with organic ligands can cross cell membranes, although
less readily than carcinogenic Cr(VI) (29).
Measurements of Cr content of biologic tis-
sues is not a trivial task even when expen-
sive instrumentation is available, as
contamination problems are ubiquitous.
Introduction of appropriate background
correction techniques in the 1980s and an
understanding of contamination sources
have led to a reliable determination of Cr in
biologic fluids by many laboratories; how-
ever, Cr measurements can still be variable
and profoundly influenced by sample
collection and laboratory procedures.

Estimation of a biologically active dose
of Cr(VI) based on chromosomal damage
end points even in highly exposed industrial
workers has not been successful (30–33),
perhaps because of frequent concomitant
exposures to other clastogens (i.e., Ni in
welders) as well as strong confounding
effects of cigarette smoking.

In an attempt to develop a biologic dosimeter for Cr(VI) exposures that would
not be influenced by dietary Cr and would
reflect damage to a biologically relevant
target DNA, investigators have applied
measurements of DNA–protein cross-links
(DPC) in peripheral blood mononuclear
cells of human subjects as a biomonitoring
tool. This paper will summarize our find-
ings in human studies and present prelimi-
nary data on biologic characteristics of
Cr(VI)-induced DPC.

**DNA–Protein Cross-Link Assay**

In the past, DPC typically have been
measured using an alkaline elution method.
Although the alkaline elution assay is quite
sensitive, its application to biomonitoring
studies was seriously hampered by the need
for freshly prepared cell lysates and by the
assay’s limited sample capacity. We have
developed a new, simple, and sensitive assay
for measurements of DPC, which has been
validated using various cross-linking agents
in cultured cells as well as in chromate-
exposed animals (34). This new assay is
more sensitive in the detection of Cr(VI)-
induced DPC in experimental animals than
the previously used alkaline elution method
(35). The principle of the assay is based on
the selective precipitation of proteins and
protein-linked DNA in the presence of
sodium dodecyl sulfate (SDS) and K+. The
percentage of total cellular DNA precipi-
table by K-SDS is a measure of DPC.
DNA was quantitated with the fluorescent
dye Hoechst 33258 (Sigma Chemical Co.,
St. Louis, MO) and with this dye a minimal
amount of 1 million cells per sample
was required to detect cross-linked DNA.
We found that the DPC remained stable
when SDS-lysed cell samples were kept
frozen. This allowed us to store samples and
use internal standards to correct for differ-
ences in the determination of DPC in sepa-
rate assay runs (batch effect). The minimal
sample size needed to detect a statistically
significant increase in DPC without the
use of internal controls was calculated to
be seven controls and seven exposed indi-
viduals (36). The detection limit for DPC
was estimated to be about 1 adduct per
1–2 × 10^7 bases. Two independent studies
devoted to the technical aspects of the K-
SDS DNA–protein cross-linking assay
demonstrated its high reproducibility in
different laboratories (37,38). The most
important factor in the reproducible
measurements of DPC by this assay was
effective shearing of SDS-lysed cell samples
(37). Although the K-SDS assay cannot be
considered specific for Cr-induced DPC,
we were able to eliminate many aldehyde-
duced cross-links by heating of cell lysates
prior to the cross-link analysis (39).

**Measurements of DNA–Protein Cross-Links in Human Populations**

The potential utility of DPC measurements
in assessment of human exposure to toxic
Cr compounds has been tested in different
groups of individuals who were either occupa-
tionally exposed to Cr(VI) or who lived in
Cr-contaminated areas (summarized in
Table 1). Our first study group included
metal-arc welders (mild-steel welders) ex-
periencing exposure to Cr via inhalation of
welding fumes (40). The data demonstrated
a significant increase in DPC in the total
white blood cells of welders as compared to
local controls. It should be noted that mild-
steel welders are considered a relatively low
Cr-exposure group. The increase in DPC in
the welder group might have been more sig-
nificant had we used lymphocytes in place
of total white blood cells. Approximately
60% of total white blood cell population are
granulocytes that stay in the bloodstream
only for a few days; therefore, these cells
could not experience significant Cr expo-
sure. Significant increases in the number of
lymphocytic DPC among welders were also
reported by other investigators (41,42).

DPC were also measured in lympho-
cytes of highly exposed chrome-platers
and two groups of control subjects from
Bulgaria (43). Individual internal doses
of Cr were estimated on the basis of Cr mea-
surements in urine, erythrocytes, and lymph-
cytes. Lymphocytic DPC correlated strongly with Cr levels in erythrocytes at
low and moderate exposures; however, no
 correlation was found with urinary Cr

| Population | Cr exposure, source | Cr exposure, level | DNA–protein cross-links | Confounders | Reference |
|------------|---------------------|-------------------|------------------------|-------------|----------|
| Welders    | Welding fumes       | Low–medium        | Increased              | None        | Costa et al. (40) |
|            |                     |                   |                        | Popp et al. (41) |
| Residents of Hudson County, New Jersey | Environmental | Low | Increased | None | Taioli et al. (44) |
| Residents of Jambol, Bulgaria | Environmental | Low–medium | Increased | None | Zhitkovich et al. (43) |
| Chrome platers, Jambol, Bulgaria | Acidic chromium mists | High | No increase above environmentally exposed controls | None | Zhitkovich et al. (43) |

*All increases in DPC were statistically significant.*
measurements. Urinary Cr levels represent not only exposure to Cr (VI), but also to dietary Cr(III), whereas red blood cell Cr levels are indicators of exposure to toxic hexavalent Cr. At the highest levels of Cr exposure, DPC leveled off. The saturation of lymphocytic DPC was estimated to occur at 7 to 8 µg/l Cr in erythrocytes. A number of control subjects exhibited significantly high internal Cr doses apparently due to environmental exposures, and this was associated with elevated levels of DPC.

The use of DPC measurements in lymphocytes for assessment of biologically effective doses of Cr was additionally tested in the United States among individuals experiencing environmental Cr exposure (44). Hudson County, New Jersey, was a major center for chrome and bichromate production for about 70 years (22). Approximately 2 million tons of Cr-containing slag were generated. The slag was used as clean fill in a large number of residential, commercial, and industrial construction sites. Studies conducted by the New Jersey Department of Environmental Protection (Trenton, NJ) and Rutgers University (Piscataway, NJ) have registered increases in spot urinary Cr concentrations and in dust Cr levels in local homes (21,23). In collaboration with Rutgers University, we obtained blood from individuals thought to have Cr exposure based on elevated urinary and house dust Cr levels. There was a substantially higher level of DPC among these individuals as compared to control subjects from other areas of New Jersey and New York that were not contaminated with Cr (44).

In all human studies DPC values were not affected by weight, age, race, gender, or smoking among both control and exposed populations (40-46).

New Developments in DNA-Protein Cross-Link Assay
The sensitivity of the DPC assay was directly related to the methodology of DNA determination. The most sensitive technique of DNA detection involves hybridization with 32P-labeled DNA probes. Figure 1 compares the results of DPC measurements by the standard fluorescent assay and by a slot-blotting procedure using human-specific Alu DNA as a hybridization probe. Use of the hybridization technique led to an approximately 4-fold decrease in the background compared to the fluorescence-based method. As a result, the dose-response curve for chromate exhibits substantially greater sensitivity. The major reason for the observed decrease in background values with the use of the hybridization method of DNA detection is likely related to the elimination of a nonspecific signal observed in the Hoechst assay because of the presence of residual amounts of SDS. The Alu-based method enables the quantitation of small amounts of DNA on the filter and permits the use of substantially smaller number of cells for assessing intracellular DPC. Hybridization detection of DPC can allow measurements of these adducts in as few as 1000 to 2000 cells, making DPC possible in cell targets such as nasal epithelium or buccal cells. It is possible that if this improved DPC assay had been applied in our human studies, the differences between control and exposed populations might have been significantly greater.

Mutagenicity of Amino Acid- and Glutathione-DNA Cross-Links
An important consideration in the application of biomarker measurements to risk assessment is understanding the biologic consequences of a measured end point. The role of a biomarker in risk assessment might be more significant if the biomarker measures biologic changes that are mechanistically involved in the pathogenesis of chemical-induced disease. For DNA-based bioassays, the mechanistic involvement in the pathogenesis is usually related to the question of mutagenicity of a particular DNA modification. To study the mutagenic potential of Cr(VI)-induced DPC, we tested mutagenicity of amino acid-/glutathione-Cr(III)–DNA adducts using a shuttle vector mutagenicity system (47,48). In addition to the formation of DPC, chromate exposure of cells also leads to DNA cross-linking of tripeptide glutathione and several individual amino acids (18). Both protein and amino acid-/glutathione–DNA cross-links induced by chromate are mediated by Cr(III) and formed by involvement of the same chemical groups (49,50). The pSp189 plasmid containing sup F gene as a mutagenic target (47) was treated with Cr(III) or preincubated Cr(III)–glutathione mixtures, purified from unreacted components and then used for human cell transfection experiments. Adducted and control plasmids were transfected into normal human fibroblasts immortalized with SV40 virus (HF/SV cell line). Two days after transfection the plasmid was isolated and treated with DpnI restriction to eliminate unreplicated molecules. The DpnI-resistant plasmids were transfected into an indicator bacteria and the frequency of mutant colonies was determined (Figure 2). The results show that bulky glutathione–Cr(III)–DNA adducts were strongly mutagenic, whereas small Cr(III)–DNA adducts were only weakly mutagenic. Cr(III)–histidine and Cr(III)–cysteine adducts had intermediate mutagenic potential. A significant increase in the mutation frequency was observed at less than 1 glutathione adduct per 100 base pairs. Because they are substantially larger than DNA–glutathione adducts, DPC might have an even greater mutagenic potential. Unfortunately, similar studies with DPC are challenging with the current technology. A complete account of experiments on the mutagenicity of Cr(III)–DNA adducts is published elsewhere (51).

Stability of Chromate-Induced DNA-Protein Cross-Links
The ability of the DPC biomarker to detect low-level human exposures to Cr could indicate that this type of DNA damage might not be efficiently repaired. Unrepaired DPC would then accumulate in long-lived populations of lymphocytes.
Human T lymphocytes, which represent more than 90% of total lymphocyte population, are estimated to have a life span of several years (52). Because repair of chromate-induced DPC has not been well studied, we attempted to assess persistence of these adducts in human Burkitt lymphoma cells transformed with Epstein–Barr virus (EBV-BL cells). Transformation of cells with Epstein–Barr virus leads to the inactivation of apoptotic DNA fragmentation. Intactness of the DNA is essential to the determination of DPC by the K-SDS assay, and the use of cells with inactivated DNA fragmentation pathway permits uncompromised analysis of DPC over long periods of time. Figure 3 shows results of DPC measurements in EBV-BL cells exposed to two concentrations of potassium chromate. Cells were exposed for 3 hr and DPC were measured at different time intervals after chromate removal. Levels of DPC increased with time and these adducts do not seem to be repaired. At 100 μM chromate exposure, DPC appeared to rise almost linearly with the duration of incubation. This dose of chromate did not induce detectable cytotoxicity at any time points studied, as determined by the trypan blue staining test, and 48 hr after exposure to 100 μM chromate, cell proliferation was restored. The highest dose of chromate did not induce cytotoxicity immediately after exposure, but 48 hr later only 50% of the cells were alive, which coincided with a decrease in the levels of DPC. Relatively weak cytotoxicity of short-term chromate treatments was also reported for CHO cells (53,54).

Similar, though not identical, kinetics of DPC formation were observed when primary human lymphocytes were treated with chromate (Figure 4). Levels of DPC were again rising with time, with the most significant increases observed between 24 and 48 hr. No cytotoxicity was observed immediately or 3 hr after chromate exposure, whereas at the 48-hr postincubation point cell viability was decreased to 60 to 70%. Although a larger number of samples and lower doses of Cr(VI) will be needed to better define the time course of chromate-induced DPC, the results already obtained point to an accumulation of DPC with time. Although these results may appear unusual based on kinetics of adduct formation by organic compounds, Cr-dependent DNA adduction might be very different. Once formed inside the cells, Cr(III) is slowly excreted, with a substantial portion of this metal essentially trapped intracellularly. The long-term presence of Cr(III) presents a possibility of additional formation of DPC with time until Cr(III) is completely inactivated by chelation with other ligands.

**Summary**

The application of the K-SDS method for measurements of DPC in peripheral blood lymphocytes appeared to provide a sufficiently sensitive approach to determine DNA damage in populations environmentally or occupationally exposed to Cr. The ability to detect low levels of Cr exposure with the use of DPC seems to be related to the kinetics of the formation of this adduct. Experiments with amino acid and peptide Cr(III)–DNA cross-links suggested that Cr-induced DPC were likely to be mutagenic in human cells. The K-SDS assay is relatively nonspecific with respect to the chemical nature of DPC; however, only a limited number of agents induce a thermostable form of cross-links and some of these compounds do not reach the bloodstream to damage lymphocytic DNA (exemplified by no elevation of cross-links in smokers). The detection of elevated levels of DPC in several human populations with relatively low Cr exposures, as well as a strong correlation between internal dose...
of Cr and measurable adducts, indicated that determination of DPC can be used as a test for the presence of DNA damage due to Cr exposure. Given that the cross-link assay is inexpensive and permits analysis of many samples, we envision that DPC measurements could be performed for rapid DNA-damage screening of potentially exposed populations, whereas additional, more complex Cr-specific analyses that are being developed will be done only for the cross-link-positive groups or individuals.

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