Monitoring Human Lymphocytic DNA–Protein Cross-links as Biomarkers of Biologically Active Doses of Chromate

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A simple and sensitive assay for DNA–protein cross-links has been used as a biomarker of chromate exposure and early carcinogenic effects. Pilot studies of DNA–protein cross-links in peripheral blood lymphocytes have been conducted with individuals who had higher exposure to chromate, including welders, and with individuals who had lower levels of exposure such as residents living in a chromium-contaminated area in Jersey City, New Jersey. Studies were also conducted in two Bulgarian cities (Jambol and Burgas) with different levels of air pollution and Cr(VI) exposure and in chrome platers in Bulgaria who had high exposure to chromate. DNA–protein cross-links in U.S. welders and in individuals living in Hudson County, New Jersey around chromium-contaminated areas were significantly higher compared to matched controls. Although blood and urinary levels of chromium were not extensively studied in these populations, we were able to obtain these measurements in the Bulgarian population. Chromium levels in red blood cells of controls living in Burgas were in the order of 1 to 2 ppb chromium, and these individuals had the lowest levels of DNA–protein cross-links. However, the chromium levels in Jambol ranged from about 2 to 7 ppb in red blood cells of city residents to about 22 ppb in chrome platers. DNA–protein cross-links were saturated at about 7 to 8 ppb chromium in the red blood cells, and cross-links correlated well only with chromium levels in red blood cells. Urinary chromium levels did not correlate well with either DNA–protein cross-links or chromium levels in with red blood cells. — Environ Health Perspect 104(Suppl 5):917–919 (1996)

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Introduction

Despite substantial evidence of the carcinogenic activity of hexavalent chromium compounds, it is still widely used in industrial situations (*1*). Hexavalent chromium compounds can be found in cement and is used as an antirust agent in water cooling systems (*1*). Workers become exposed to it during welding and chrome plating (*1*). There are also areas where rocks rich in Cr(VI) have been used as landfill, such as in Hudson County, New Jersey (*2*). Hexavalent chromium is a major contaminant in many Superfund sites (40% of all sites).

Epidemiological studies have demonstrated a strong association of occupational exposure to chromate particles in chrome plating and refinery workers and the induction of nasal and respiratory cancers (*1,3*). Hexavalent chromium-containing compounds have been shown to induce respiratory cancers in experimental animals as well (*3*). Hexavalent chromium-containing compounds are active in all genotoxic and mutagenicity assays (*1,3*). The potent genotoxic and carcinogenic activity of hexavalent chromium is due to its rapid uptake into cells (*4*). Hexavalent chromium exists as an oxyanion at physiological pH and is actively taken up by an anion transport system, which is present in all mammalian cells. In contrast, the trivalent form of chromium is taken up 1000-fold less readily than hexavalent chromium compounds, and, in general, trivalent chromium complexes exhibit about 1000-fold lower toxic activity in cellular systems compared to hexavalent chromium (*3,4*).

Intracellularly, many of the genotoxic effects of hexavalent chromium are attributable to its reduction to trivalent chromium (*5–9*). There are some unique chemical features of Cr(VI)→Cr(III) reduction system in cells. For example, trivalent chromium forms octahedral complexes involving six ligands of coordination. The binding of Cr(III) to various ligands is considered very stable. However, during the conversion of hexavalent chromium to trivalent chromium, oxygen radicals are generated and chromium forms various intermediates, such as Cr(V) and Cr(IV), which exhibit different reactivities from Cr(III) (*10–12*). This creates a unique set of reactions that is not characteristic of those occurring with Cr(III) alone. The Cr(V) and Cr(IV) intermediates are unstable and eventually form Cr(III) in cells (*10–12*). A major intracellular reducer of hexavalent chromium to trivalent chromium is ascorbic acid, but glutathione and other reducing agents can also catalyze these reactions (*13*).

Studies conducted in our laboratory have demonstrated that trivalent chromium ultimately participates in the cross-linking of proteins to DNA (*8*). Although DNA–protein cross-links (DPC) represent a lesion that can be detected in cells after exposure to hexavalent chromium compounds, they are not the only DNA lesion induced by chromate (*14*). For example, DNA–DNA cross-links involve trivalent chromium (*14*). Trivalent chromium can also complex amino acids and glutathione to DNA (*15*). In fact, complexes of amino acids and glutathione with Cr(III) accounted for about 50% of all Cr(III) DNA adducts and are considerably more abundant than complexes of proteins cross-linked with DNA (*16*). However, the detection of protein–DNA cross-links can be accomplished with a simple and sensitive assay, whereas other types of Cr(III) DNA adducts cannot be as easily detected.

In this report, we summarize the data that we have obtained on studies of DPC

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Abbreviations used: DPC, DNA–protein cross-links; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

as a biomarker of human exposure to chromate. We present a preliminary report on our Bulgarian studies, which will be published in more detail elsewhere.

Materials and Methods

DNA–protein cross-links were assessed by the method of Zhitkovich and Costa (*17*), as recently modified (*18*). Peripheral blood mononuclear cells were isolated by a standard Ficol sodium diatrizoate protocol using Histopaque-1077 (Sigma). The isolated mononuclear cells were washed in phosphate buffered saline, counted and 2×10^6 cells were lysed in 0.5 ml of 0.5% sodium dodecyl sulfate (SDS), 20 mM Tris–HCl (pH 7.5). The lysed samples were placed at –70°C until analyzed for DPC.

Samples were thawed at 37°C and the DNA was sheared by passage of the cell lysates 4 times through a 21-gauge needle. The lysate was expelled into a tube by applying high pressure and 0.5 ml of 100 mM KCl, 20 mM Tris (pH 7.5) was then added. The content was mixed by vortexing the tube for 5 sec at maximum speed, and the tubes were then heated for 10 min at 65°C. Samples were removed from the water bath, inverted 3 times, and then placed on ice for 5 min to form a K-SDS precipitate. The precipitate was collected by centrifugation at 6000*g* for 5 min at 4°C. The supernatant was removed and the pellet was resuspended in 100 mM of KCl, 20 mM Tris–HCl (pH 7.5) by brief vortexing at the highest setting. The samples were again heated at 65°C for 10 min and the washing steps, as well as the heating steps described above, were repeated 2 more times. Protein linked to DNA was released from the final potassium dodecyl sulfate precipitate by treatment with 0.2 mg/ml proteinase K in a 0.5-ml solution containing 100 mM KCl, 20 mM Tris–HCl (pH 7.5), and 10 mM EDTA. The samples were then incubated at 50°C for 3 hr. At this point the samples were stored at –70°C. Fifty microliters of 4 mg/ml bovine serum albumin (BSA) was added to each tube and the samples were placed on ice. The tubes were centrifuged at $12{,}000\times g$ for 10 min at 4°C, and the supernatant was taken to determine the quantity of DNA. It is important at this point to avoid disturbing the potassium dodecyl sulfate pellet while aspirating the supernatant because dodecyl sulfate interfered with the Hoechst 33258 assay.

DNA was detected using Hoechst 33258. DNA standards were prepared at concentrations of 100, 200, 500, 1000, 2000, and 5000 ng/ml. We took 0.5 ml of a standard DNA or the entire supernatant from the potassium dodecyl sulfate pellet and mixed it with 0.5 ml of freshly prepared Hoechst dye reagent, 250 ng/ml. Precaution was taken to avoid exposure to light at this point, and the samples were placed in the dark for 10 min. Fluorescence was measured by setting excitation wavelength at 365 and emission wavelength at 450 to 460 nm. The DNA standard curve and samples were read for fluorescence. A BSA tube was used as the standard blank. The percent of DNA precipated was taken as a measure of DPC (*17,18*).

DNA–protein cross-links have been examined in various populations. The exposed and control groups had similar demographic characteristics, except that all exposed were males and five of the controls were females. All individuals recruited for this study were white. Exposed and control groups were similar with respect to alcohol consumption and smoking status. No consumption of vitamins, hobbies or second jobs associated with chromium exposure was reported. A more detailed account of the method for the Bulgarian studies will be published in the future.

Results

We previously examined DPC in individuals engaged in mild steel welding, which results in a much lower level of chromium-exposure than stainless-steel welding. The results of these studies showed a 58% increase in cross-links in the cases compared with controls, yielding a level of significance of $p=0.01$ (*18*). We have also found higher DPC among residents of Hudson County, New Jersey, who lived around chromium-containing mine tailings (*19*). In this study, control ($n=49$) levels of cross-links were 0.8 ± 0.4, while exposed subjects had levels of 1.3 ± 0.5 ($n=33$) ($p<0.001$) (*19*). However, chromium exposure in these studies has not been documented, and the question of actual human exposure to chromate remains in doubt. Similarly, there is uncertainty as to the cause of the elevated DPC.

Recently, we measured airborne, red and white blood cell, and urinary chromium concentrations to estimate actual human exposure to chromium. Red blood cell chromium is currently considered the best indicator of hexavalent chromium exposure. Figure 1 shows the excellent correlation between lymphocyte levels of DPC and chromium concentrations in red blood cells among controls in Bulgaria ($r=0.62$; $p=0.008$). These individuals lived in two cities, Jambol or Burgas, which experience strikingly different air pollution. There was a lack of correlation between urinary and red blood cell chromium for controls and chrome platers (data not shown). Similarly, there was also a lack of correlation between urinary chromium in controls or chrome platers with lymphocyte DPC (data not shown). In contrast, there was an excellent correlation between red blood cell chromium and DPC among two cities with different air pollution, as well as among chrome platers (Figure 2). This figure illustrates the relationship of red blood cell chromium and DPC using a

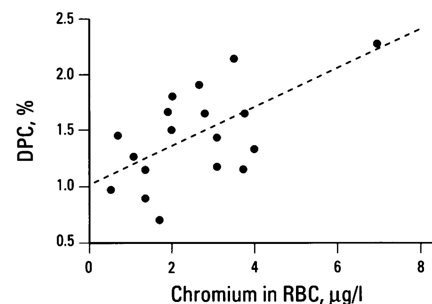

Figure 1. Correlation of chromium in red blood cells (RBC) with lymphocyte levels of DNA–protein cross-links (DPC). Chromium was measured in RBC using graphite furnace atomic absorption. DPC were measured in lymphocytes of these same individuals. Shown are controls that lived in Burgas or Jambol, Bulgaria.

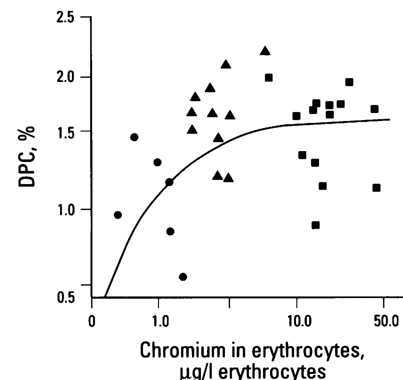

Figure 2. Correlation between chromium in red blood cells and DNA–protein cross-links (DPC). Chromium was measured in red blood cells of residents of Burgas ($n=6$) and Jambol ($n=11$) and of chrome platers ($n=14$) using graphite furnace atomic absorption. DPC were also determined in lymphocytes of these same individuals. The equation fitting the mode is $y=1.7$ $(1-e^{1.4(\text{chromium in erythrocytes})})$. Data in graph are log transformed. ●, Burgas; ▲, Jambol; ■, chrome platers.

nonlinear model. The DPC response appeared to have obtained its maximum level at about 7 to 8 ppb chromium levels in red blood cells (Figure 2), and it did not increase further when levels of chromium were higher in red blood cells, such as in chrome platers from Bulgaria. The plateau level as shown by the mathematical model occurred with a DPC of 1.7%. A more detailed account of these data will be published later.

Discussion

It is extremely important to relate the response of a biomarker of exposure and effect to the actual internal dose. In the case of chromium, the exposure parameters are quite complex because this element is both essential and toxic. The essentiality of the trivalent form of chromium is not well understood. It is thought to be involved in the transport of glucose in cells and the action of insulin on this process. However, dietary trivalent chromium represents a substantial contribution to urinary chromium. This is perhaps why measurement of urinary chromium may not be a reliable way to monitor low-level exposure to hexavalent chromium. Hexavalent chromium is actively transported into cells and remains there for the life of a cell, whereas trivalent chromium does not readily enter cells.

Our results clearly show that the red blood cell levels of chromium correlate well with lymphocyte DPC. These two parameters, however, do not correlate with urinary chromium levels. These findings suggest that urinary chromium levels may not be a good way to monitor human exposure to hexavalent chromium. Measurement of red blood cell levels of chromium is sometimes problematic due to the interference with a biological matrix. This problem can be circumvented by using O_2 infusion with graphite furnace atomic absorption during the ashing procedure because this will yield a more complete combustion and eliminate any possible matrix interference.

An additional important implication of DPC is that they not only assess exposure to chromate or other agents inducing such cross-links, but it measures a biologically active dose. This goes beyond just measuring the metal level, since the presence of a metal in a cell does not indicate biological activity. Metals are stored as inactive complexes in cells, and their presence does not necessarily constitute a biological effect. It is difficult to say with certainty whether the increase in DPC was due to chromium or some other chemical. Analysis of DPC showed that they are very reliable biomarkers that is not confounded by variables such as sex, race, weight, or mild smoking (*20*). However, issues concerning the persistence of DPC and DNA repair need to be addressed.

A number of agents produce DPC, including *cis*-platinum, formaldehyde, and UV light. However, UV light and formaldehyde are not likely to cause increased levels of cross-links in peripheral lymphocytes. Other unknown environmental factors may also be involved in elevating lymphocyte DPC.

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