Comparison of DNA Lesions and Cytotoxicity Induced by Calcium Chromate in Human, Mouse, and Hamster Cell Lines

Masayasu Sugiyama, Xin-Wei Wang, and Max Costa

ABSTRACT

DNA lesions, cytotoxicity, and cellular uptake of CaCrO₄ were compared in Chinese hamster ovary, in mouse embryo fibroblast C3H10T½, and in human osteosarcoma cells. The concentration of CaCrO₄ that reduced colony formation by 50% was 2-3-fold less in human osteosarcoma cells than in C3H10T½ or Chinese hamster ovary cells. Alkaline elution studies showed that CaCrO₄ induced DNA single strand breaks in a concentration dependent manner in all three cell lines. However, the human cells exhibited four times more breaks than the hamster cells and two times more than C3H10T½ cells when the CaCrO₄ exposure conditions were equivalent. Alkaline elution studies also demonstrated the formation of DNA-protein cross-links by CaCrO₄ in all three cell lines. In hamster and mouse cells the induction of these DNA-protein cross-links was dependent on concentrations that ranged from 5 to 50 μM for 6 h; however, the cross-links were saturated at 25 μM in human cells and at 50 μM in mouse and hamster cells. The level of cross-links was four times greater in the human cells compared to the mouse cells and was a factor of 2 greater in the hamster cells compared to the mouse cells. The uptake of CaCrO₄ was linear with respect to time and concentration. Uptake of CaCrO₄ was equivalent in the human and mouse cells, but was a factor of 4 less in the hamster cells. The single strand breaks were almost entirely repaired after an 18-h incubation in metal free medium in all three cell lines, whereas DNA-protein cross-links persisted in these cell lines in proportion to their initial levels. These results demonstrate differences in the sensitivity of human, hamster, and mouse cells to CaCrO₄, and suggest that the repair-resistant DNA-protein cross-link may be important in mediating the long term toxic and carcinogenic effects of CaCrO₄.

INTRODUCTION

Chromium compounds have been shown to cause tumors and to produce a number of toxic effects in both humans and animals (1-3). Chromate potently induces transformation and mutation in cultured mammalian cells of hamster (4-6) and human (7) origin. Chromium compounds have been shown to induce sister chromatid exchanges and chromosomal aberration in cell lines of human (7-10), mouse (11), and hamster (5, 6, 9, 10, 12-15) origin. In cultures of human (9, 16-18), as well as mouse (17), and hamster cells (19-21), the carcinogen chromate induced DNA single strand breaks and DNA-protein cross-links. However, there have been only a few studies that compared the toxic effects of chromium in cultured cells originating from different species (9, 22). In fact, these studies have illustrated that chromate was more toxic to the human cells than to the hamster cells. Therefore, it is important to further assess whether there are significant differences in the toxic action of metal ions such as chromate among cells originating from different species.

In the present study we have compared the cytotoxicity, cellular uptake, and DNA damage induced by CaCrO₄ in human, mouse, and hamster cell lines, utilizing the alkaline elution technique. Our findings illustrate that the human cells were the most sensitive of the cell lines examined in terms of induction of DNA damage and cytotoxicity. These results are explained, in part, by the greater uptake of chromate in the human cell lines, but these uptake studies cannot entirely explain the differences observed.

MATERIALS AND METHODS

Chemicals. The radioisotope [¹⁴C]deoxythymidine (58 mCi/mmol) and Na₂CrO₄ were obtained from New England Nuclear (Boston, MA) and ICN Chemical Co. (Irvine, CA), respectively. Free acid EDTA, and sodium dodecyl sulfate were acquired from Sigma Chemical Co. (St. Louis, MO). Tetrapropyl ammonium hydroxide (10% aqueous solution) was purchased from RSHA Chemical Co. (Ardsville, NY). Polycarbonate filters were from Nucleopore (Pleasanton, CA), and polyvinyl chloride filters were from Millipore Corp. (Bedford, MA). Proteinase K was obtained from EM Laboratories, Inc. (Elmsford, NY). Bovine serum and α-minimal essential medium were from Hazleton, Inc. (Denver, PA), and trypsin was obtained from Gibco, Inc. (Grand Island, NY). Liquisint was acquired from National Diagnostics, Inc. (Somerville, NJ).

Cell Culture. The AA8 CHO, 10T½, and HOS cells were maintained in a humidified atmosphere of 95% air-5% CO₂ at 37°C in α-minimal essential medium supplemented with 10% fetal bovine serum and a 1% solution of penicillin-streptomycin-Fungizone (Gibco). 10T½ or HOS cells used in all experiments were at passages 12-19 or 49-59, respectively. HOS cells were a generous gift from Dr. D. Blair (National Cancer Institute, Frederick, MD).

Alkaline Elution. The alkaline elution technique for analysis of DNA lesions was performed as described by Kohn et al. (22) with minor modifications. Cells were seeded into 100-mm diameter tissue culture dishes and incubated for 48 h with [¹⁴C]thymidine (0.02 μCi/ml). Logarithmically growing cells were washed and incubated in complete growth medium in the absence of radioactivity for 2 h prior to treatment with CaCrO₄. Cells were treated with CaCrO₄ in complete growth medium or SGM consisting of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer at pH 7.2 containing (mM) NaCl, 100; KCl, 5; CaCl₂, 2; and glucose, 5. Following treatment, cells were washed twice and removed from the monolayer by scraping with a rubber policeman into ice cold Puck's saline A (5 mM NaHCO₃, 6 mM glucose-5 mM KCl-140 mM NaCl, pH 7.2). At this stage the cytotoxicity was assessed by trypan blue exclusion. To study DNA damage by alkaline elution a volume of 4-5 ml containing 0.5-1.0 x 10⁶ cells was deposited onto 25-mm polycarbonate filters (when DNA was assayed for strand breaks) or polyvinyl chloride filters (when DNA was assayed for cross-links). Filters were rinsed with ice cold saline A and the cells were then lysed directly onto the filters by passage of 5 ml of 2% sodium dodecyl sulfate solution containing 0.025 M EDTA. DNA was eluted at a flow rate of 0.028 ml/min with 25 ml of a solution that contained both 0.025 M EDTA and 2% tetrapropyl ammonium hydroxide (pH 12.15). Fractions...
of approximately 2.5 ml were collected and radioactivity of these fractions was determined by scintillation counting after the addition of 7 ml of Liquiscint and 0.7 ml of glacial acetic acid. The filters were digested for 1 h at 60°C in 0.4 ml of 1 N HCl and neutralized with 2.5 ml of 0.4 N NaOH at 25°C for 1 h. Radioactivity in the filters was then determined by scintillation counting in 10 ml of Liquiscint containing 1.0 ml of glacial acetic acid. The degree and nature of DNA cross-links were assessed by utilizing a test dose of X-rays (300 rads) and proteinase K as previously described (15). To quantify the extent of DNA single strand breaks and DNA-protein cross-links, the strand scission factor (SSF) and cross-link factor (CLF) were calculated from the alkaline elution patterns according to the following relationship: $SSF = \frac{\log A}{\log B}$, $CLF = \frac{\log A - \log AX}{\log A - \log BX}$, where $A$ is the amount of DNA retained in the sixth fraction of untreated sample and $B$ is the DNA retained in the sixth fraction of the CaCrO$_4$-treated samples; $AX$ is the amount of DNA retained in the sixth fraction of untreated X-irradiated cells, and $BX$ is the amount of DNA retained in the sixth fraction of CaCrO$_4$-treated X-irradiated cells (23).

Colony-forming Assay. Following treatment with CaCrO$_4$, cells were rinsed twice with fresh medium and then trypsinized. Cell number was determined with a Coulter counter or a hemocytometer. An appropriate number of cells (200–1200) were then plated into culture dishes and allowed to form colonies. Cells were fixed with 95% ethanol and stained with a 0.2% crystal violet solution. The number of surviving colonies (>50 cells) in each plate was expressed as a function of the original number of cells plated. This survival was then compared to that of untreated cells. Plating efficiencies for untreated CHO cells ranged from 95 to 105%, while normal 10T$^\text{1/2}$ cells had plating efficiencies ranging from 11 to 19%, and HOS cells plated with an efficiency of 28–32%.

Cellular Uptake of $^{51}$Cr. Cells were incubated with 2 $\mu$Ci of $^{51}$Cr at various concentrations, then washed twice with SGM, and dislodged from the dishes by scraping. The cell number was determined, and the cellular uptake of $^{51}$Cr was estimated by the radioactivity detected in a Beckman gamma counter.

RESULTS

Measurement of DNA Single Strand Breaks in Various Cell Lines. Logarithmically growing CHO, 10T$^\text{1/2}$, and HOS cells were treated with CaCrO$_4$, and DNA single strand breaks were measured by the alkaline elution method. Fig. 1 shows that CaCrO$_4$ induced different amounts of DNA single strand breaks in each of the three cell lines after they had been exposed to CaCrO$_4$ in either a SGM or complete culture medium. At equivalent CaCrO$_4$ concentration, HOS cells exhibited a greater degree of strand breakage based upon the elution kinetics illustrated in Fig. 1. 10T$^\text{1/2}$ cells were intermediate while CHO cells exhibited the least sensitivity (Fig. 1). Fig. 2 shows the effect of concentration on the induction of strand breaks by CaCrO$_4$ in the CHO cells. As the exposure concentration increased there was more strand breakage. In agreement with the results shown in Fig. 1, HOS cells exhibited the most breaks at all concentrations, while 10T$^\text{1/2}$ cells were intermediate and CHO cells were the least sensitive.

Assessment of DNA-Protein Cross-Links in Various Cell Lines. Fig. 3 shows the concentration-dependent effects of CaCrO$_4$ on the induction of DNA-protein cross-links in CHO cells which is measured by the increased retention of DNA from cultures treated with CaCrO$_4$ and irradiated with a test dose of 300 rads. Since the treatment of all cell lysates with proteinase K totally reversed the observed cross-links, it was presumed that the increased retention of DNA was due to DNA-protein cross-links and not to DNA-DNA cross-links (not shown). CHO and 10T$^\text{1/2}$ cells had increased DNA-protein cross-links when the concentration of CaCrO$_4$ was raised to 50 $\mu$M; however, these cross-links reached a maximum in HOS cells at 25 $\mu$M, while CHO and 10T$^\text{1/2}$ cells required 50 $\mu$M to attain a maximal level of cross-links. At equivalent exposure conditions, CaCrO$_4$ induced more DNA-protein cross-links in CHO cells than in 10T$^\text{1/2}$ cells (Fig. 3). The HOS cells exhibited the most sensitivity in the CaCrO$_4$ induction of DNA-protein cross-links (Fig. 3).

Uptake of Chromate in Various Cell Lines. In an attempt to understand the differences in the effects of CaCrO$_4$ among the cell lines, the uptake of this metal was examined. Fig. 4 shows that the uptake of chromate increased linearly with treatment time (1–24 h) as well as concentration (25 and 50 $\mu$M). With respect to time and concentration the uptake of chromate was the least in CHO cells but was of an equivalent level in 10T$^\text{1/2}$ and HOS cells. Table 1 shows the cellular uptake of chromate was two to three times greater in SGM than in complete tissue culture growth medium.

Cytotoxicity of CaCrO$_4$ in Various Cell Lines. Fig. 5 examines the survival of the three cell lines treated with various concentrations of CaCrO$_4$ for 6 h in complete tissue culture medium. HOS cells were the most sensitive to CaCrO$_4$, while 10T$^\text{1/2}$ cells were intermediate, and CHO cells were the least sensitive. A 50% survival level was found for HOS, 10T$^\text{1/2}$, and CHO cells at CaCrO$_4$ concentrations of 15, 30, and 45 $\mu$M, respectively. There were no colonies formed in HOS cells 10 to 12 days following treatment with 50 $\mu$M CaCrO$_4$. The viability of CaCrO$_4$ treated or untreated cells was equivalent based upon trypsin blue exclusion studies. There was also initially little effect of CaCrO$_4$ on cell numbers even at levels that strikingly reduced plating efficiency (i.e., 50 $\mu$M CaCrO$_4$ in HOS cells for 6 h).

Repair of DNA Lesions in Various Cell Lines. Fig. 6 illustrates the repair of single strand breaks and DNA-protein cross-links...
Fig. 2. Comparison of the effect of CaCrO₄ concentration on the extent of DNA single strand breaks in various cell lines. HOS, 10T½, and CHO cells were treated for 6 h with CaCrO₄ in complete growth medium. Following treatment, the cellular DNA was analyzed by alkaline elution as illustrated in Fig. 1. The DNA single strand breaks induced by CaCrO₄ were expressed as strand scission factor. Bars, SD. a, P < 0.01 compared to 10T½ or CHO cells. b, P < 0.05 compared to CHO cells (Student's t test).

at 18 h following removal of CaCrO₄. Note that essentially all the single strand breaks were repaired during this time interval, whereas the DNA-protein cross-links persisted in proportion to their initial levels. In contrast to single strand breaks there was apparently no repair of DNA-protein cross-links.

DISCUSSION

Previous studies demonstrated that treatment of cultured mammalian cells with chromium compounds resulted in DNA single strand breaks and DNA-protein cross-links but not in DNA interstrand cross-links (17, 21). In the present study, the DNA lesions and cytotoxicity of CaCrO₄ were compared in three different cell lines of human, mouse, and hamster origin. CaCrO₄ induced a different level of both DNA single strand breaks and DNA-protein cross-links in each of these three cell lines.
DNA DAMAGE INDUCED BY CaCrO₄

![Graph showing DNA damage induced by CaCrO₄](image)

Fig. 6. Repair of DNA lesions induced in various cell lines by CaCrO₄. HOS, 10T½, and CHO cells were treated for 6 h with CaCrO₄ in complete growth medium. Cellular DNA was analyzed by alkaline elution after an additional 18-h incubation in CaCrO₄-free medium. DNA single strand breaks and DNA-protein crosslinks were expressed as strand scission factor and cross-link factor, respectively. Bars, SD. *P < 0.05 compared to 10T½ or CHO cells.

These findings are consistent with those in the literature showing that human cells were more sensitive to sister chromatid exchange, to DNA single strand breaks, as well as to the cytotoxicity when compared with hamster cells (9, 22). We have extended these preliminary findings by conducting a more thorough examination of DNA lesions with the alkaline elution technique and by assessing metal uptake as well as cytotoxicity in three different cell lines. While single strand breaks and cytotoxicity studies indicated that the following could be ranked in decreasing order of sensitivity: (a) HOS, (b) 10T½, and (c) CHO cells; the uptake of chromate was equivalent in HOS and 10T½ cells but was lower in CHO cells. The volume and surface area of each of these cells may, partially, explain the observed differences in the metal uptake, since HOS cells were smaller than 10T½ cells, but both of these cells were much larger than CHO cells. For example a confluent monolayer of CHO cells in a 100-mm tissue culture plate may contain about 20 × 10⁶ cells, while a similarly confluent monolayer plate of HOS cells could contain 10–15 × 10⁶, and the maximal concentration of 10T½ cells that a 100-mm plate would hold is 4-6 × 10⁶ cells. Differences in the size of cells is also obvious at the level of light microscopy. A major factor for these differences is probably related to spreading of the cells onto the monolayer and not so much to large differences in cell volume. The larger more spread out cells will have a greater surface area for chromate exposure and this may have contributed to the amount of chromate uptake. CaCrO₄ was not as readily taken up by the cells in normal tissue culture growth medium as it was in a SGM, but this may be due to the inhibitory effects of ligands such as amino acids that bind chromate and prevent it from entering cells.

It is well known that chromate which contains hexavalent chromium can enter the cells by the sulfate transport system, and it is then reduced to the trivalent form by intracellular reductive metabolism perhaps involving the microsomal enzyme system (24, 25). Since in vitro experiments show that only the trivalent form of chromium can form a stable ternary metabolic perhaps involving the microsomal enzyme system, it is then reduced to the trivalent form by intracellular reductive metabolism perhaps involving the microsomal enzyme system (24, 25).

Since in vitro experiments show that only the trivalent form of chromium can form a stable ternary complex with DNA and protein (17, 25), the formation of DNA-protein cross-links may strongly depend upon this reductive metabolism. Consequently the differences observed in the DNA-protein cross-link levels may be due to such a reductive metabolism whose affinity or capacity might vary with each cell type.

Since there was no survival of HOS cells at 50 µM CaCrO₄, it was possible that this lack of dependency of DNA-protein cross-links on the concentration of CaCrO₄ may be due to cell death, as well as to the loss of cells containing DNA cross-links or the limited cellular uptake of chromium at toxic levels. However, since HOS cells treated with CaCrO₄ exhibited linear uptake of chromate at 25 and 50 µM and there was no difference in viability of cells by trypan exclusion test or in cell growth immediately following CaCrO₄ exposure, the former possibilities remain unlikely. It is possible that at the highest concentration of chromate used, the DNA or protein may have become saturated with chromium ions, resulting in an absence of reactive sites for the formation of DNA-protein cross-links. However, there was still a dose dependency in the induction of single strand breaks at concentrations where cross-links were saturated. Previous studies with CHO cells have also shown that the formation of DNA-protein cross-links required time, whereas strand breaks were at a maximal level immediately following treatment and could always be directly related to the concentration of CaCrO₄ (21). In the present study, the order of sensitivity to DNA-protein cross-links in the cell line examined was not consistent with the sensitivity to the formation of CaCrO₄ induced single strand breaks. Other studies have shown that DNA single strand breaks induced by chromium compounds were associated with the cellular levels of glutathione and cytochrome P-450, whereas protein cross-links were not dependent upon the levels of these components (26). These results suggest that DNA-protein cross-links may be formed by chromium by a different mechanism from that of the single strand breaks. Since DNA-protein cross-links were saturated at different levels in each cell line, it was important to test a number of concentrations when DNA-protein cross-linking activity of CaCrO₄ was compared in each cell type.

Recently, we have reported that CaCrO₄ induced DNA damage as well as cytotoxicity in CHO cells predominantly during the early S phase of the cell cycle (21). Transcriptionally active euchromatic DNA is known to be replicated during early S phase. Previous clastogenic studies have also showed that chromium compounds induced the highest frequency of sister chromatid exchanges during the early S phase of the cell cycle of human cells (8). Sen and Costa (15) have shown that a majority of CaCrO₄ induced sister chromatid exchanges were not localized in heterochromatic regions of CHO chromosomes. Therefore, it was possible that the sites of chromium interaction with euchromatin may differ in each of the cell types examined. The differences in sensitivity to CaCrO₄ of the three cell lines examined may also be due to other factors such as their tissue of origin.

The frequency of chromate induced single strand breaks correlated with its cytotoxicity in each of the three cell lines. However, DNA single strand breaks in all three cell types were repaired after removal of CaCrO₄. In contrast, DNA-protein cross-links were not repaired and, in fact, increased in proportion to the concentration of CaCrO₄. Therefore, cytotoxicity of CaCrO₄ may be associated with onset of repair-resistant DNA-protein cross-links.

Since the frequency of mutation and transformation is related to DNA damage present during the period of DNA replication, persistent DNA lesions which are not easily repaired would, therefore, have the greatest impact in permanently altering DNA structure and function. DNA-protein cross-links in all
three cell lines resisted repair even at low noncytotoxic concentrations, whereas single strand breaks were repaired at all concentrations. These findings suggest that the DNA-protein cross-link is a very important lesion that may permanently alter the process of genetic transfer.

ACKNOWLEDGMENTS

The authors would like to thank Kitty Goldstein for excellent secretarial assistance and Dr. P. Sen for her criticism of this manuscript.

REFERENCES

Comparison of DNA Lesions and Cytotoxicity Induced by Calcium Chromate in Human, Mouse, and Hamster Cell Lines

Masayasu Sugiyama, Xin-Wei Wang and Max Costa


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/9/4547

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.