The carcinogen chromate causes DNA damage and inhibits drug-mediated induction of porphyrin accumulation and glucuronidation in chick embryo hepatocytes

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Abstract
DNA damage by chromate in chick embryo hepatocytes has been correlated with the effect of chromate on inducible cell functions. Treatment of chick embryo hepatocytes with chromium(VI) in the form of sodium chromate resulted in the rapid uptake of chromate and the induction of DNA lesions in a time- and concentration-dependent manner. DNA interstrand cross-links, strand breaks and DNA-protein cross-links, as measured by the alkaline elution technique, were observed after treatment of the hepatocytes with chromate concentrations (2.5 - 10 \(\mu\)M) which did not affect cell viability. The effect of chromate on inducible cell functions was measured by assaying propylisopropylacetamide-induced accumulation of porphyrin and glucuronidation of phenol red by intact cells. Chromate inhibited propylisopropylacetamide-induction of porphyrin accumulation and phenol red glucuronidation in a time- and concentration-dependent manner which paralleled DNA damage. DNA damage was removed and inducibility of porphyrin accumulation by propylisopropylacetamide plus deferoxamine methanesulfonate was restored 21 h following a 2 h pretreatment with chromate. Chromium(III) in the form of chromic nitrate at concentrations (2.5 - 10 \(\mu\)M) which did not affect the viability of the cells. This is the first demonstration of chromate-induced interstrand cross-links in cultured cells, although this type of DNA lesion has been observed \(in vivo\) (10,11). The effect of chromate on inducible cell functions in the hepatocytes at these concentrations was determined by observing the effect of chromate treatment on the subsequent induction of phenol red glucuronidation and of accumulation of porphyrin caused by propylisopropylacetamide (PIA*) (12,13). These tests are much more sensitive to the effects of chromate on cell functions than are the normal cell viability tests (vide infra). The observed inhibition of induced cellular functions by chromate was found to correlate with the chromate-induced DNA damage.

Materials and Methods

Chromatography

Chromium(VI) in the form of sodium chromate has been shown to be tumorigenic in animals and mutagenic in various bacterial and mammalian cell systems (2). However, carcinogenic and mutagenic effects have not been demonstrated with chromium(III). An 'uptake-reduction' model has been proposed to explain the genotoxicity of chromium(VI) (3). Chromate, the form of chromium(VI) at physiological pH, readily enters cells, whereas the cell membrane is relatively impermeable to chromium(III) complexes. Once inside the cell, chromium(VI) is reduced ultimately to chromium(III) by cellular components such as the microsomal electron-transport cytochrome P-450 system (4). Chromium(III) which is produced upon reduction of chromium(VI) is the form ultimately bound to cellular constituents including DNA and protein (3).

The relationship between damage to DNA caused by chromium(VI) compounds in cells and the effect of chromium(VI) on inducible cell functions has not been previously examined. In human skin fibroblasts chromium(VI) produced DNA single-strand breaks which were detectable by alkaline sucrose gradients at toxic concentrations (0.5 - 5 mM) only (5). Other studies (6-8) with cultured cells have not determined the toxicity of chromium(VI) or the effect of chromium on inducible cell functions under the conditions where DNA damage was observed.

Since metabolism of chromium(VI) is important to its reaction with DNA, we have used the highly metabolically active chicken embryo hepatocytes to study chromate-induced DNA damage. Using the alkaline elution technique (9), DNA interstrand cross-links, DNA-protein cross-links and DNA strand breaks were observed in chick embryo hepatocytes treated with concentrations (2.5 - 10 \(\mu\)M) of chromate which did not affect the viability of the cells. This is the first demonstration of chromate-induced interstrand cross-links in cultured cells, although this type of DNA lesion has been observed \(in vivo\) (10,11). The effect of chromate on inducible cell functions in the hepatocytes at these concentrations was determined by observing the effect of chromate treatment on the subsequent induction of phenol red glucuronidation and of accumulation of porphyrin caused by propylisopropylacetamide (PIA*) (12,13). These tests are much more sensitive to the effects of chromate on cell functions than are the normal cell viability tests (vide infra). The observed inhibition of induced cellular functions by chromate was found to correlate with the chromate-induced DNA damage.

Materials and Methods

Chemicals

Bovine serum albumin (BSA) and 3,3',5-triiodothyronine (T\(_3\)) hormone were obtained from Sigma Chemical Co. (St. Louis, MO). Williams E Medium (WE) was obtained from Flow Laboratories, Inc. (McLean, VA). Fetal bovine serum (FBS) was obtained from Gibco Laboratories, (Grand Island, NY). Dexamethasone (DEX) was obtained from Dell Laboratories, (Tanneck, NJ). DES was obtained from Ben Venue Laboratories, (Bedford, OH). Sodium chromate was obtained from Fisher Scientific, (Pittsburgh, PA). PIA was donated by Hoffmann-La Roche, (Nutley, NJ). All other chemicals were reagent grade. Preparation and treatment of cultured chick embryo hepatocytes

Hepatocytes were prepared from the liver of 16-day embryos and cultured as described in detail previously (14). After the initial cell isolation, the hepatocytes were resuspended in WE containing 1 \(\mu\)g insulin/ml, 0.3 \(\mu\)g DEX/ml, and 1.0 \(\mu\)g T\(_3\)/ml. For alkaline elution, chromate uptake, and porphyrin accumulation experiments, \(-2 \times 10^8\) cells were seeded into sterile
Assay for release of lactate dehydrogenase

The uptake of chromate by the chick embryo hepatocytes was monitored by the loss of chromat from the media. The absorption spectrum of the chromium(VI) in media showed an absorbance maximum at 372 nm indicating that the species in solution was chromate. The media alone did not have an absorption maximum in the region of chromate absorbance. Figure 1 shows that the amount of chromate in the media decreases dramatically with time in the presence of cells. The time course for the percentage decrease of chromate concentration in the media was similar for the different initial concentrations (5-10 µM). The uptake of chromate was very rapid since approximately one quarter of the initial chromate concentration was lost from the media within 30 min. At the end of 2 h only 22-39% of the initial concentration of chromate remained. Incubating chromat with fresh media or with media that had been exposed to cells for 2 h had no effect on the chromat concentration. These results indicate that chromat is rapidly taken-up by the hepatocytes.

Fluorometric assay for DNA

The amount of DNA eluted and the amount remaining on the filter was determined using the microfluorometric assay reported previously (10,15) with the following modifications. Filters were removed from the elution columns, placed into test tubes, and air dried. To each fraction, a 400 µl aliquot of a 2 mg/ml solution of BSA was added, followed by an appropriate volume of ice-cold PBS. After the PBS had drained, the cells were immediately lysed with 3 ml of 2% SDS lysis solution. The elution was carried out as described previously (10) with the exception that cells which were irradiated received 660 Rads of X-ray. Calculations for DNA lesion frequency were based on equations derived by Kohn (9) which use values of chromate concentration in the media was similar for the different initial concentrations (5-10 µM). The uptake of chromate was very rapid since approximately one quarter of the initial chromate concentration was lost from the media within 30 min. At the end of 2 h only 22-39% of the initial concentration of chromate remained. Incubating chromat with fresh media or with media that had been exposed to cells for 2 h had no effect on the chromat concentration. These results indicate that chromat is rapidly taken-up by the hepatocytes.
Chromate-induced DNA damage in chick hepatocytes

Significant DNA damage was first observed 15 min after initiating exposure of the cells to chromate (data not shown). Figure 2 shows typical elution curves obtained with hepatocytes treated with chromate. Upon treatment of hepatocytes with chromate both strand breaks (faster elution compared with control in unirradiated samples) and cross-linking (slower elution compared with control in irradiated samples) were observed. Since chromate caused DNA strand breaks under conditions where it also caused DNA cross-links, the equations of Kohn et al. (9) were used to calculate the cross-link frequency. Treatment of the hepatocytes with chromate resulted in DNA cross-links which generally increased with chromate concentration and with time of exposure (Table I and Figure 3). Following a 2 h treatment with chromate, incubation of cells for 21 h in the absence of chromate resulted in a dramatic decrease in the amount of cross-links (Table I and Figure 3). A low but significant amount of cross-linking persisted even though strand breaks were almost completely removed (see below) during this period. No DNA cross-links were observed after treatment of the hepatocytes with chromium(III) in the form of chromic nitrate for 1 h at concentrations up to 25 times (250 \(\mu\)M) those used with chromate (Table I).

The nature of the DNA cross-links was examined by determining the sensitivity of the lesions to proteinase K digestion (9). DNA-protein cross-links are removed by this treatment, whereas interstrand cross-links are unaffected (9). After treatment of lysates with proteinase K significant DNA interstrand cross-linking was observed (Table I and Figure 3) in cells treated with 2.5—10 \(\mu\)M chromate. An increase in interstrand cross-linking was observed between 30 min and 2 h exposure (Table I). The amount of DNA interstrand cross-linking increased with increasing concentration of chromate (Figure 3). In addition to DNA cross-links, DNA strand breaks were induced by treatment of hepatocytes with chromate (Table I). Strand breaks analyzed by proteinase K treatment increased dramatically with chromate concentration but increased only slightly with time of exposure at all concentrations tested (Table I and Figure 4). In contrast to the slight increase in
strand breaks observed between 30 min and 2 h exposure to chromate using proteinase K digestion, the amount of DNA strand breaks detected in the absence of proteinase K treatment decreased from the 30 min to the 2 h exposure time (Table I). The amount of strand breaks affected by proteinase treatment increased from <25% at 5—10 μM concentrations of chromate after 30 min exposure (Table I) to >60% of the strand breaks after a 2 h exposure (Table I and Figure 4). Incubation of the hepatocytes in the absence of chromate for a further 21 h following a 2 h exposure to chromate resulted in removal of strand breaks (Table I and Figure 4). No difference between strand breaks assayed in the presence and absence of proteinase K remained under these conditions (Table I and Figure 4). No DNA strand breaks were observed after treatment of the cells for 1 h with chromium(III) nitrate at concentrations up to 25 times those used with chromate (Table I).

**Chromate inhibition of PIA-induced phenol red glucuronidation and porphyrin accumulation**

PIA is the most effective phenobarbital-like inducer of cytochrome P-450, porphyrin accumulation and phenol red glucuronidation in cultured chick embryo hepatocytes (12,18,19). The accumulation of porphyrin is due to increased induction of β-aminoolevulinate (ALA) synthase (13). Glucuronidation of phenol red, the pH indicator in the medium, is an indication of the increase in UDP-glucuronyltransferase activity (12,18,19). The effect of pre-incubating hepatocytes with chromate on the subsequent induction of porphyrin accumulation and phenol red glucuronidation by PIA was used as a criterion for the effect of chromate on inducible cell functions. These assays are particularly useful since the induction of these activities appears to involve both transcription (20) and translation (20,21).

The induction of phenol red glucuronidation by PIA was inhibited by pretreating the cells with chromate (Figure 5A,B). Pretreatment of hepatocytes with chromate

![Fig. 4. Concentration dependence of chromium-induced DNA strand breaks 2 h after exposure of chick embryo hepatocytes to sodium chromate.](http://dx.doi.org/10.1093/carcin/05.8.962)
Chromate-induced DNA damage in chick hepatocytes

Fig. 5. The effect of preincubating chick embryo hepatocytes with various doses of chromate on the subsequent inducibility of medium phenol red glucuronidation caused by PIA. Conditions for seeding and plating of the hepatocytes are the same as those described in the legend to Figure 1 with the following modifications. PIA (20 µg/ml) was added to the cells (seeded in 6.0 cm plates) after removal of chromate and the addition of fresh WEH. The cells were then incubated for an additional 24 h to allow induction of glucuronyl transferase. Glucuronidation of phenol red was measured as described in Materials and Methods. (A) The concentration- and time-dependent effects of chromate on induction of phenol red glucuronidation. (●), 2 h exposure to chromate; (△), 1 h exposure to chromate; (●), 30 min exposure to chromate. In the absence of chromate the phenol red glucuronide concentration was 8.2 µM for control cells (uninduced) and 16.1 µM for PIA-treated cells. (B) The effect of chromium(III) and the effect of chromate in the presence and absence of FBS on the PIA-induced glucuronidation of phenol red. (●), 2 h exposure to chromium(III). In the absence of chromium(III) phenol red glucuronide concentration was 4.2 nM for control cells (uninduced) and 11.3 nM for PIA-treated cells. (●), 2 h exposure to chromate in the absence of FBS; (○), 2 h exposure to chromate in the presence of FBS. In the absence of chromate phenol red glucuronide concentration was 5.1 nM for control cells (uninduced) and 13.8 µM for PIA-treated cells. The error associated with duplicate values was ± 3%.

inhibition of PIA-induced glucuronidation (53% versus 40%) at 10 µM chromate, but was ineffective (2.4% versus 3.3%) at 25 µM (Figure 5B).

Chromate, in a time- and concentration-dependent manner, inhibited the induction of total porphyrin accumulation caused by treating cells with both PIA and DES (Figure 6). DES, by chelating iron, inhibits formation of heme, the feedback regulator of ALA synthase (13). DES was used in these studies to increase porphyrin accumulation and thereby, make the porphyrin assay more sensitive. Pretreatment of cells for 2 h with concentrations of chromate ranging from 2.5 to 10 µM resulted in decreasing levels of porphyrin accumulation (Figure 6). Incubation of the hepatocytes for 24 h in fresh media following a 2 h treatment with 5, 7.5 or 10 µM chromate completely restored the ability of cells to be inducible for porphyrin accumulation (Figure 6). Little or no decrease in PIA-induced porphyrin accumulation was seen after incubation of the cells for one hour with chromium(III) at doses ranging from 5 to 250 µM (data not shown).

As an indication of cell viability (14) release of lactate dehydrogenase (LDH) from hepatocytes was measured. No increase in LDH release was observed from hepatocytes treated with concentrations up to 10 µM chromate for 2 or 18 h (data not shown). Furthermore, as in the experiment presented in Figure 6, there was no increase in LDH release after a 2 h exposure to chromate followed by a medium change then 6 h exposure to PIA and DES (data not shown).

Observation of cells by phase contrast microscopy confirmed the results obtained with LDH. No changes were visible in cells which were treated with chromate at concentrations up to 10 µM for up to 18 h. In contrast, a 2 h exposure of the cells to 25 µM chromate, a concentration which totally inhibited glucuronidation of phenol red (see Figure 5B), caused a marked contraction of the colonies, still seen 24 h after removal of the chromate.

Discussion

Many studies indicate that chromate requires metabolism for activation. The microsomal electron-transport cytochrome P-450 system has been shown to possess chromate-reductase activity in vitro (4) and may be important in producing DNA-damaging chromium species. Therefore, cultured chick embryo hepatocytes are a useful cell system for studies of the mechanism of chromium(VI) carcinogenicity since the cells do not replicate, are inducible for cytochrome P-450, and have metabolic activities similar to those of intact animals (12,18,19).

The rapid disappearance of chromate from the culture media indicates that chromate was readily taken up by the hepatocytes. Facile cellular uptake of chromium(VI) has been observed in many different types of cells (22—28). Chromate...
Chromate induced DNA damage in the form of DNA strand breaks, DNA-protein cross-links and DNA-interstrand cross-links in various cultured cell systems. The cytotoxicity of the anti-tumor agents, cis-dichlorodiammineplatinum(II) and chloroethylnitrosourea, has been correlated with their ability to induce DNA interstrand cross-links in various cultured cell systems. Different repair mechanisms appear to be important in removing DNA cross-links induced by chromate. (8), cis-dichlorodiammineplatinum(II) (31) and chloroethylnitrosourea (32). Rat liver and chick hepatocytes appear to have repair systems which efficiently remove chromate-induced DNA lesions and perhaps make liver less sensitive to the deleterious effects of chromate which have been observed in lung and kidney tissues (1,33). It is possible that other types of chromate-induced DNA lesions, e.g., intrastrand cross-links or double strand breaks, which are not detectable by the alkaline elution technique, may be responsible for the toxicity of chromate. Additional studies are needed to determine the persistence of DNA cross-links seen upon chromium(VI) treatment of various cells and whole animals suggests that these lesions may be related to the carcinogenicity of chromium(VI) compounds. The cytotoxicity of the anti-tumor agents, cis-dichlorodiammineplatinum(II) and chloroethylnitrosourea, has been correlated with their ability to induce DNA interstrand cross-links in various cultured cell systems. Different repair mechanisms appear to be important in removing DNA cross-links induced by chromate. Rat liver and chick hepatocytes appear to have repair systems which efficiently remove chromate-induced DNA lesions and perhaps make liver less sensitive to the deleterious effects of chromate which have been observed in lung and kidney tissues (1,33). It is possible that other types of chromate-induced DNA lesions, e.g., intrastrand cross-links or double strand breaks, which are not detectable by the alkaline elution technique, may be responsible for the toxicity of chromate. Additional studies are needed to determine the.
The DNA damage in chick hepatocytes correlated well with the effects of chromate on cell function as measured by the inhibition of PIA-induced phenol red glucuronidation and porphyrin accumulation. Chromate inhibited the induction of phenol red glucuronidation and porphyrin accumulation by PIA at concentrations and times where extensive DNA damage was observed. Inducibility of porphyrin accumulation by PIA plus DES was completely restored by allowing cells to recover from chromate treatment for 21 h before addition of the inducing agents. Similarly, DNA damage was almost completely repaired during this period since only a small amount of DNA cross-linking and strand breaks persisted.

It has been shown that PIA induces cytochrome P-450, phenol red glucuronidation and porphyrin accumulation in chick hepatocytes (12, 14, 18). Cytochrome P-450 and phenol red glucuronidation were induced in a parallel manner in the chick hepatocytes and it was concluded that a common mechanism may be responsible (12). The increase in glucuronidation of phenol red can be ascribed to an increase in UDP-glucuronosyltransferase. In both rats (34, 35) and chicken embryos (39), phenobarbital induced mRNAs for cytochrome P-450. In rats phenobarbital has also been shown to induce mRNAs for NADPH-cytochrome c oxidoreductase and epoxide hydratase (36, 37) but not for cytochrome b5 (37). It is likely that the cytochrome P-450 and UDP-glucuronosyltransferase genes are under the control of the same operon in chick hepatocytes and that PIA induction results in the synthesis of mRNAs for both enzymes. PIA also induces porphyrin accumulation which is a measure of the increase in ALA synthase (the rate determining enzyme in liver heme biosynthesis) in the chick hepatocytes (18, 38). Protoporphyrin accumulates because of the very low activity of ferrochelatase in these cells (38). Decrease in available iron, caused by DES treatment, further enhances porphyrin accumulation (13). Induction of ALA synthase in chick embryo liver cells has also been shown to be dependent on the synthesis of mRNA (20).

Since chromate damage to DNA is apparent under conditions where it inhibits PIA induction, it is possible that the chromium-induced DNA lesions adversely affect mRNA synthesis. The chromate could also affect mRNA processing and transport from the nucleus, as well as translation of the message. However, Bianchi et al. (30) found that chromium(VI) (19 - 190 μM) inhibited RNA synthesis to a much greater extent than protein synthesis in BHK cells. In BHK cell cultures, K₂Cr₂O₇ (10 - 100 μM) inhibited RNA synthesis up to 30% under conditions where no inhibition of protein synthesis was observed (22). Additional studies are needed to determine the mechanism of action of chromate on PIA induction of phenol red glucuronidation and porphyrin accumulation in chick hepatocytes.

In conclusion, the chick embryo hepatocytes appear to be a good system for studying chromate-induced DNA damage and repair, and chromate-induced changes in cellular functions.

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References