Down-regulation of the DNA-repair endonuclease 8-oxo-guanine DNA glycosylase 1 (hOGG1) by sodium dichromate in cultured human A549 lung carcinoma cells

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Hexavalent chromium is a genotoxic human pulmonary carcinogen that elevates DNA oxidation, apparently through the generation of reactive DNA-damaging intermediates including CrV, CrIV and reactive oxygen species. We tested the hypothesis that elevation of DNA oxidation may also be through inhibition of the expression of the repair glycosylase for 8-oxo deoxyguanine (hOGG1) in cultured A549 human lung epithelial cells. Treatment with sodium dichromate (0–100 µM, 16 h) resulted in a concentration-dependent decrease in the levels of OGG1 mRNA as measured by both RT–PCR and RNase protection assay. Sodium dichromate at 25 µM and above gave a marked reduction of OGG1 mRNA expression which was not seen at 1 µM and below. No effect on the expression of the apurinic endonuclease hAPE or the housekeeping gene GAPDH was observed at any of the concentrations of sodium dichromate investigated. Treatment of cells with the pro-oxidant H2O2 (0–200 µM) for 16 h had no detectable effect on the levels of OGG1 mRNA or protein expression suggesting that the effect of sodium dichromate is not mediated by H2O2. Western blotting demonstrated that sodium dichromate (100 µM; 16 h and >25 µM; 28 h) markedly reduced levels of OGG1 protein in nuclear cell extracts. Additionally, treatment of cells with sodium dichromate (>25 µM; 28 h) resulted in a concentration-dependent decrease in the ability of nuclear extracts to nick a synthetic oligonucleotide containing 8-oxo deoxyguanine (8-oxo dG). We conclude that the elevation of 8-oxo dG levels observed in A549 cells treated with sodium dichromate may be, at least in part, due to a reduced capacity to repair endogenous and hexavalent chromium-induced 8-oxo dG.

Introduction

Hexavalent chromium (CrVI) is known to induce lung cancer (1) and is genotoxic in a number of in vitro systems causing DNA-strand breaks (2,3), DNA–DNA cross links (4), DNA–protein cross links (5) and Cr-DNA adducts (6). Although the mechanism of CrVI-genotoxicity remains to be fully elucidated, intracellular reduction of CrVI by cellular antioxidants to reactive intermediates such as CrV (7), CrIV (8) and possibly reactive oxygen species (ROS) including HOO-, O3, O2- and H2O2 (9–11) is believed to be important. Such reductive processes cause intracellular oxidation a priori. It is hypothesized that the resulting oxidative stress is central to many of the cellular effects of hexavalent chromium, being not only responsible for DNA damage but also for chromium-mediated changes in gene expression, including induction of nuclear transcription factor-kB (NF-kB) (8,12), haem oxygenase-1 (HO-1) (13) and activation of p53 (10,11). In addition, oxidative stress has also been implicated in chromium-dependent activation of mitogen-activated protein kinases (14). Cellular targets of ROS are numerous and include lipids, proteins and DNA (15–17). Support for a role of DNA oxidation comes from the findings that CrV and possibly CrIV are able to oxidize nucleotides and DNA (18,19). One of the principal lesions produced in DNA following oxidative stress is 8-oxo 2-deoxyguanine (8-oxo dG), which as a result of mis-pairing to adenine during DNA-replication results in the formation of G to T transversions, a commonly observed mutation in the gene of the tumour suppressor p53 in human cancers including lung cancer (20–23). CrVI and CrIII have been demonstrated to cause 8-oxo dG adducts in isolated DNA (24,25). Recently, we have demonstrated that culture of human A549 lung epithelial cells with sodium dichromate (10 µM) results in elevated levels of both 8-oxo dG as determined by immuno-cytocchemistry and DNA-strand breaks introduced by formamidopyrimidine (FaPy) DNA-glycosylase (comet assay), known to be involved in the repair of this lesion (2). These findings support the hypothesis that formation of 8-oxo dG may represent an important mechanism by which hexavalent chromium initiates lung cancer.

As a consequence of the mutagenic potential of 8-oxo dG, cells have evolved a complex repair system termed the GO-system (26) whose function is to recognize and repair 8-oxo dG. In eukaryotic cells one of the key components of this system is 8-oxo guanine-DNA glycosylase 1 (OGG1). OGG1 cleaves the glycosidic bond of 8-oxo dG preferentially at 8-oxo dG:C base pairs (27). The phosphodiester bond at the resulting apurinic (AP)-site is cleaved by β-elimination and the process of base excision repair completed by the sequential action of apurinic endonuclease, DNA polymerase β and DNA-ligase III. Chemical induction of OGG1 considered to be mediated by ROS has been demonstrated in certain in vivo (28) and in vitro (29) systems but not others (30). In the current study we investigated mRNA and protein expression, and activity of OGG1 in human alveolar epithelial A549 cells exposed to sodium dichromate in vitro to determine whether CrVI is able to modulate expression of hOGG1, either by induction via ROS or by inhibition and consequently contributing to the observations of elevation of 8-oxo dG.

Materials and methods

Cell culture

Human lung carcinoma A549 cells (European Cell Culture Collection No. 86012804) were grown to 100% confluency in T25 (Falcon) culture flasks at 37°C in a humidified, 5% CO2 atmosphere using DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Abbreviations: CrVI, hexavalent chromium; ROS, reactive oxygen species; HO-1, haem oxygenase-1; NF-kB, nuclear transcription factor-kB; CBP, cAMP-responsive element-binding protein-binding protein; 8-oxo dG, 8-oxo 2-deoxyguanine; FaPy, formamidopyrimidine.

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0.1 mg/ml streptomycin. Fetal bovine serum was included in the medium for all treatments.

**ATP and TUNEL assays**

To investigate the possible effects of sodium dichromate (0–500 µM, 16 h) on cellular ATP-levels and the frequency of apoptosis, A549 cells were grown to confluency in 12-well plates (Nunc, Naperville, US) and chamber slides (Nunc, Lab-Tek II) respectively. Following treatment, intracellular ATP-levels were measured using an ATP-bioluminescent kit according to the manufacturer’s instructions (Sigma, FL-AA, Dorset, UK). The frequency of apoptosis was assessed by the TUNEL method using a commercially available kit (Promega, Southampton, UK).

**RNA isolation and RNase protection assay**

Total cellular RNA was isolated using a Nucleospin RNAII kit (Clontech, Basingstoke, UK) according to the manufacturer’s instructions. hOGG1 (495 bp), hAPE (419 bp) and GAPDH (304 bp) cDNA were synthesized by reverse transcription (RT)–PCR carried out on 0.1 µg of RNA using a Superscript One-step RT–PCR kit (Invitrogen, Paisley, UK) according to the manufacturer’s instructions. Primers, hOGG1 (forward, 5′-atcgttgtcccaaccaac-3′; and reverse 5′-cagcagataagcccaacag-3′), hAPE (forward, 5′-atagctgtagatgtagatg-3′; and reverse 5′-caacactttgagtagca-3′) and GAPDH (forward, 5′-aagggagctagctagcatg-3′; and reverse, 5′-tccacacctgcttgtag-3′) were synthesized by MWG Biotech UK. cDNA was purified using quipspin columns (Ambion, Texas, US). Biotinylated anti-sense RNA probes were synthesized from purified cDNA using a Liq’n nuclease PCR cloning kit (Ambion) followed by in vitro transcription with a maxiscript kit (Ambion, Texas, US) and biotin-UTP (Sigma) according to the manufacturer’s instructions. RNase protection assays were carried out on 10 µg of total RNA using a RPAIII kit (Ambion). Protected fragments were resolved on a 5% acrylamide–urea gel and transferred to positively charged nylon membrane (Brightstar Plus, Ambion) and bands detected by chemiluminescence (Brightstar, Ambion). Quantification of OGG1 mRNA relative to GAPDH was carried out by densitometry using the Scion software package. The results of the RNase protection assay were further supported by semi-quantitative RT–PCR using 0.1 µg of RNA and a previously published PCR cycle (28). Levels of hOGG1 mRNA were assessed relative to GAPDH albeit without quantification relative to an internal standard.

**Preparation of nuclear protein extracts**

Cells in lysis buffer (0.6% NP-40, 150 mM NaCl, 10 mM HEPES pH 7.5, 1 mM EDTA) supplemented with protease inhibitors (10 µl/mM mammalian protease inhibitor cocktail, Sigma) were incubated on ice for 20 min and the nuclei pelleted by centrifugation at 4°C (15 min, 3000 g). The nuclear pellet was homogenized in high salt buffer (25% v/v glycerol, 420 mM NaCl, 20 mM HEPES pH 7.5, 0.2 mM EDTA, 1.5 mM MgCl₂, 0.5 mM DTT, containing 10 µl/mM mammalian protease inhibitor cocktail, Sigma) and the lysate incubated on ice for 30 min. Following centrifugation (18 000 g, 30 min, 4°C) the supernatant was retained for analysis.

**Western blotting**

Nuclear extracts (10 µg) were resolved on a 10% SDS–polyacrylamide gel, transferred to PVDF membrane (Amersham Pharmacia Biotech) and blocked overnight at 4°C (TBS–0.05% Tween 20, 5% low fat milk). Membranes were incubated with polyclonal rabbit anti-OGG1 (1:500 dilution, Novus Biologicals) in blocking buffer (25% v/v glycerol, 420 mM NaCl, 20 mM HEPES pH 7.5, 1.5 mM MgCl₂, 0.5 mM DTT, containing 10 µg/mll mammalian protease inhibitor cocktail, Sigma) and the bands detected using chemiluminescence detection (Amersham Pharmacia Biotech).

**Endonuclease nicking assay**

A single-stranded 24 mer (15 pmol) containing 8-oxo dG at position 10 (Trevigen, Gaithersburg, US) was 5′-end labelled with γ-32P-ATP (NEN) using a DNA 5′ End-Labeling System (Promega) according to the manufacturer’s instructions and un-incorporated label removed by centrifugation through a G-10 spin column (Sigma). The end-labelled oligomer was annealed to a 1.2-fold excess (18 pmol) of a complementary 24 mer (Trevigen) by heating to 95°C for 15 min followed by gradual cooling to room temperature. Endonuclease nicking assays were performed in 10 mM Tris–HCl pH 7.5, 100 mM KCl, 10 mM EDTA containing 1 µg of nuclear protein and 1 pmol of the annealed oligomer at 37°C for 1 h. Reactions were terminated by the addition of loading buffer (95% formamide, 0.5 mM EDTA, 0.02% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol) and heating to 95°C for 5 min followed by cooling on ice. Samples were resolved on 20% polyacrylamide, 8 M urea gels and bands visualized by exposing to X-ray film (Kodak, Herts, UK).

**Results**

**Cytotoxicity**

No statistically significant decrease in intracellular ATP levels were observed following treatment with either sodium dichromate (0–100 µM) or H₂O₂ (0–200 µM) for 16 h in cultured A549 cells (Figure 1A and 1B). In contrast, treatment with 500 µM sodium dichromate resulted in a statistically significant (P < 0.01) decrease in ATP levels. There was also no evidence that sodium dichromate (16 h, 0–500 µM) induced apoptosis in confluent A549 cells as assessed by the TUNEL assay (<0.01% cells apoptotic). As a positive control, incubation of cells with DNase resulted in >95% labelling index (images not shown). Based upon these results, sodium dichromate (0–100 µM) and H₂O₂ (0–200 µM) were chosen to investigate treatment-mediated changes in hOGG1 gene expression.

**Sodium dichromate decreases OGG1 mRNA and protein expression**

Initial experiments showed that sodium dichromate (0–100 µM, 2 and 4 h) had no detectable effect on the expression of OGG1 at either the mRNA or protein level as determined by RT–PCR and western blotting respectively (data not shown). To determine the effect of longer-term treatment with hexavalent chromium on the expression of OGG1 mRNA, cells were
Inhibition of \(\text{hOGG1}\) expression by sodium dichromate

**Fig. 2.** Effect of sodium dichromate on OGG1, APE and GAPDH mRNA expression as assessed by RT-PCR. A549 cells were treated for 16 h at 37°C.

**Fig. 3.** Effect of sodium dichromate on OGG1 and GAPDH mRNA expression as assessed by the RNase protection assay. A549 cells were treated for 16 h at 37°C.

Sodium dichromate-mediated repression of OGG1 mRNA was also reflected at the protein level as assessed by western blotting. Treatment of cells with sodium dichromate (0–100 \(\mu\)M) for 16 h and (>25 \(\mu\)M) for 28 h markedly reduced detectable levels of OGG1 protein in nuclear cell extracts (Figures 4 and 5). There was no evidence for an effect on nuclear levels of OGG1 protein at concentrations of 10 \(\mu\)M and below even after 48 h (Figure 5). OGG1 protein was not detectable in the cytoplasm of either control or treated cells (Figure 4) in agreement with previous reports that OGG1 is primarily nuclear in location. Treatment with \(\text{H}_2\text{O}_2\) (0–200 \(\mu\)M, 16 h) had no detectable effect on the levels of OGG1 protein in nuclear extracts (Figure 6B).

**Sodium dichromate decreases DNA-repair capacity of nuclear protein extracts**

To ascertain whether the observed decrease in OGG1 mRNA and protein expression represents a functionally significant effect on the capacity of A549 cells to repair 8-oxo dG, we assessed the ability of nuclear cell extracts to nick a synthetic oligonucleotide containing an 8-oxo dG nucleotide. Nuclear extracts from control cells proved to be efficient at this process (Figure 7). However, pre-treatment of cells with sodium dichromate (0–100 \(\mu\)M) for 28 h resulted in a concentration-dependent inhibition in the ability of nuclear extracts to cut the 8-oxo dG containing synthetic oligonucleotide (Figure 7).

**Discussion**

ROS and consequently 8-oxo dG are continually produced in cells either by normal cellular metabolism or by exposure to a wide range of physical and chemical agents. 8-oxo dG paired to cytosine in DNA is recognized and repaired by a DNA-
OGG1 mutations show a spontaneous mutator phenotype in carcinogenesis. The apurinic endonuclease APE has been reported to be induced by ROS-generating systems such as $\text{H}_2\text{O}_2$, HOCl and bleomycin (49) but not alkylating agents. The mechanism of the apparent ROS-specific induction of APE remains to be established, but may be related to the presence of a NFκB consensus sequence (but lack of a functional AP-1 binding site) in the APE promoter. In the current study, sodium dichromate had no effect on the expression of APE as assessed by western blotting, suggesting that sodium dichromate-mediated inhibition of OGG1 is not dependent on $\text{H}_2\text{O}_2$. However, the possibility that $\text{H}_2\text{O}_2$ has a transient effect on OGG1 expression cannot be completely excluded. Reduction of hexavalent chromium to Cr(V) by GSH-reductase is known to result in the generation of O• and subsequently $\text{H}_2\text{O}_2$ by the action of superoxide dismutase (47). Pentavalent chromium is believed to be among the principal species responsible for chromium-mediated oxidative stress and is capable of oxidizing DNA either directly (18) or possibly via the formation of highly oxidizing Cr(V)-peroxo complexes (19). Furthermore, catalase has been demonstrated to inhibit sodium dichromate-induced DNA damage in human peripheral blood lymphocytes (48). These observations suggest that although $\text{H}_2\text{O}_2$ may be important in the mechanism of sodium dichromate-induced DNA damage, it does not appear to play a role in sodium dichromate-mediated inhibition of $hOGG1$ gene expression.

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Cu-, Zn- and Mn-superoxide dismutase in either LL24 or A549 lung cells treated with a range of concentrations of sodium dichromate (0–100 μM). The same study observed induction of HO-1 mRNA in LL24 (>50 μM) but not A549 lung cells. In our system, we observed induction of HO-1 by RNase protection and western blotting in the A549 cell line at high concentrations of sodium dichromate (100 μM) (unpublished data). The mechanism of sodium dichromate-dependent induction of HO-1 remains to be established, and may be related to the presence of the metal response element in the HO-1 promoter rather than as a result of sodium dichromate-mediated oxidative stress.

The observation that hexavalent chromium can negatively regulate gene expression is not unique. For example, Maier et al. (50) demonstrated that sodium dichromate is able to inhibit dioxin-mediated induction of cytochrome P450 1A1 and NAD(P)H quinone oxidoreductase 1, and Shumila et al. (51) have demonstrated that in A549 cells, sodium dichromate (>20 μM) inhibits NFκB-dependent TNFα induction of IL-8. The same study also demonstrated that this inhibition was not a result of reduced NFκB binding to the IL-8 promoter. Rather it was considered to be as a result of decreased interaction between the transcription factor p65 and CBP, a coactivating molecule that links enhancer-bound transcription factors (e.g. TFIIB and TATA-binding protein) to the basal transcription machinery and is essential for NFκB-enhanced transcriptional activity (52,53). G(II) (the final cellular metabolite of hexavalent chromium) is known to bind to tridentate amino acid residues and proteins (54–56); therefore G(II) may decrease the interaction of p65 with CBP by directly altering either CBP of p65 recognition sequences. Alternatively up-regulation of other transcription factors such as c-jun (51) by sodium dichromate may result in competitive or non-competitive inhibition of p65 binding to CBP. Expression of the gene for O6-methyl guanine DNA transferase is also dependent upon CBP binding (57), but it is unknown whether CBP-binding is a requirement for HOg1 gene expression. In addition, binding of the transcription factor SP1 to its cognate target DNA sequence (GC box, present in the hoGG1 promoter) is inhibited by substitution of a guanine base for 8-oxo dG (58,59). Furthermore, decreases in the expression of genes regulated by SP1 binding and considered to be related to oxidative stress, have been observed in vivo (60).

We conclude that inhibition of hoGG1 gene expression by sodium dichromate contributes to genotoxicity as a result of decreased capacity to repair endogenous and chromate-induced 8-oxo dG. It has been estimated that chromium levels up to 5 μM may be attained in the blood of chrome pigment production workers (1,61) and it is possible that higher concentrations may be achieved locally in lung tissue. Furthermore, substantially higher exposure levels have been used in animal carcinogenicity studies (1). Therefore, this process may also contribute to the mechanism of chromium-mediated lung carcinogenicity. Current work in our laboratory is aimed at understanding the molecular mechanism(s) of this inhibition.

References

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