XRCC1 Protects against Particulate Chromate–Induced Chromosome Damage and Cytotoxicity in Chinese Hamster Ovary Cells

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Received January 13, 2006; accepted March 29, 2006

Chromosome instability (CIN) is a common feature of lung cancer cells (Masuda and Takahashi, 2002; Nakamura et al., 2003). Hexavalent chromium (Cr(VI)) is a well-known human lung carcinogen, and recent data indicate that Cr(VI) tumors are characterized by CIN (Hirose et al., 2002; Takahashi et al., 2005). In particular, epidemiological and toxicological studies indicate that the particulate Cr(VI) compounds are the potent carcinogenic form inducing tumors in experimental animals and neoplastic transformation of cultured cells (IARC, 1990; Levy et al., 1986; Patierno et al., 1988). Studies of lead chromate, a prototypical particulate Cr(VI) compound, show that the particles dissolve outside the cell to produce lead (Pb) cations and chromate anions, both of which enter the cell (Wise et al., 2004). Once inside the cells, the chromate anions induce chromosome and DNA damage, growth arrest, and apoptotic cell death, while the lead ions do not appear to reach toxic levels (Singh et al., 1999; Wise et al., 1993, 2002, 2004; Xu et al., 1992). Particulate Cr(VI) compounds induce CIN manifested as chromosomal aberrations (Wise et al., 1992, 2002), but the mechanisms involved in these effects are unknown.

Particulate Cr(VI) is a potent clastogen, inducing chromosomal aberrations in different species and cell types (DeFlora et al., 1990; Wise et al., 1992, 2002). This clastogenic effect is mediated by soluble extracellular Cr(VI) ions (Xie et al., 2004). Previous data of soluble Cr(VI) in Chinese hamster ovary (CHO) cells suggest a direct relationship between DNA single-strand breaks (SSB) and chromosome damage. For example, pretreating CHO cells with vitamin E prior to chromate treatment decreased both chromosome damage and SSB but had no effect on DNA-protein cross-links (DPC) (Sugiyama et al., 1987, 1988a,b, 1991). Similarly, pretreating cells with vitamin B2 prior to chromate treatment increased both chromosome damage and SSB but had no effect on DPC (Sugiyama et al., 1987, 1989a, 1992).

Based on these data, we hypothesize that efficient repair of particulate Cr(VI)-induced SSB is necessary to protect cells from Cr(VI)-induced chromosome damage and CIN. XRCC1 is a DNA repair protein involved in repairing both direct SSB and indirect SSB generated indirectly during base excision repair (Caldecott, 2003). It serves as a scaffold connecting many of the other proteins involved in SSB repair (Caldecott, 2003). XRCC1 is recruited to SSBs by Poly ADP-ribose polymerase 1 and then facilitates the enzymatic activities of
several repair participants including: proliferating cell nuclear antigen (PCNA), DNA polymerase beta (POLβ), and poly-nucleotide kinase (PNK) (Caldecott, 2003; Fan et al., 2004; Kubota et al., 1996; Vidal et al., 2001; Whitehouse et al., 2001). XRCC1 protein is involved in the repair of Cr(VI)-induced SSB (Christie et al., 1984), and thus, the purpose of our study was to examine the importance of XRCC1 in preventing particulate chromate–induced CIN.

MATERIALS AND METHODS

Reagents and chemicals. Dulbecco’s minimal essential medium with Ham’s F-12 medium (DMEM/F12) was purchased from Mediatech (Herndon, VA). Cosmic Calf Serum was purchased from HyClone (Logan, UT). Phosphate-buffered saline 1× (PBS), Gluta-MAX, penicillin/streptomycin, sodium pyruvate and trypsin/EDTA were purchased from Invitrogen Corporation (Grand Island, NY). Demecolchicine and sodium chromate were purchased from Sigma-Aldrich (Milwaukee, WI). Tissue culture flasks, dishes, and plastic tubes were purchased from Corning Inc. (Acton, MA).

Cell lines and cell culture. Three cell lines were used in this study. CHO AA8 cells were the parental cell line and served as a wild-type control. EM9 cells were derived from AA8 cells and are known to have a C→T substitution on codon 221 forming termination codon in the XRCC1 gene and are the SSB repair-deficient cell line (Caldecott, 2003). H9T3 cells are EM9 cells complemented with the human XRCC1 gene, which reverses the SSB repair deficiency (Thompson et al., 1990). These cell lines were provided as a generous gift from Larry Thompson. The status of XRCC1 in AA8, EM9, and H9T3 was confirmed by Western blotting (data not shown). Cells were cultured in DMEM/F12 media supplemented with 15% Cosmic Calf Serum, 2mM l-glutamine, 100 U/ml penicillin/100 μg/ml streptomycin, and 0.1mM sodium pyruvate. Cells were maintained as subconfluent adherent monolayers and subcultured at least twice a week.

Lead chromate preparation. Lead chromate (Chemical Abstracts Service # 7758-97-6, American Chemical Society reagent minimum 98% purity) is used as a particulate, water-insoluble chromium salt and was suspended in acetone as previously described (Wise et al., 2002).

Cytotoxicity assay. Cytotoxicity was determined with a clonogenic assay as previously described with few modifications (Wise et al., 2002). Briefly, 90,000 cells per well were seeded in six-well dishes and allowed to grow for 24 h. The cultures were then treated with lead chromate for 24 h. At the conclusion of treatments, cells were washed with PBS, detached from the dish with trypsin/EDTA, and reseeded at a density of 200 cells/60-mm dish and allowed to grow for 5–6 days. Colonies were fixed with methanol and stained with crystal violet. Four dishes were prepared for each treatment concentration, and a minimum of three experiments were conducted for each cell line.

Chromosome preparations. Chromosome damage was evaluated as previously described with few modifications (Wise et al., 2002). Briefly, 200,000 cells per dish were seeded in 60-mm dishes and allowed to grow for 24 h. After the 24-h treatment with lead chromate, medium was removed, and cells were harvested. Demecolchicine, 0.1 μg/ml was added 1 h before the end of the treatment time. At the end of treatment, medium was collected, and cells were washed with PBS and detached from the dish with trypsin. Cells were centrifuged for 5 min at 112 g at 4°C, and the cell pellet was treated with trypsin/EDTA and resuspended in hypotonic 0.075 M KCI solution for 10 min in order to swell the cells and the nuclei. At the end of hypotonic treatment, cells were fixed in methanol:acetic acid (3:1) and dropped onto clean wet slides. Slides were allowed to air-dry for 24 h at room temperature and then stained with 5% Giemsa in Gurr’s buffer, dried, and coverslipped. We determined a mitotic index to examine the possibility that there might be a difference in expression of frequency of mitosis between the three cell lines after lead chromate exposure and found no difference (data not shown).

Chromosome analysis and scoring criteria. Clastogenesis was measured by the production of structural chromosomal aberrations, which were scored by standard criteria (Wise et al., 1992). Gaps and breaks were pooled as “lesions” as previously described (Wise et al., 1992). This is because breaks can only be unequivocally distinguished from gaps lesions if the distalacentric fragment is displaced. Thus, pooling aberrations avoids artificial discrepancies between scorers due to different perceptions of the width of a gap relative to the width of its chromatid. Accordingly, chromatid deletions and achromatic lesions were pooled as chromatid lesions, while isochromatid deletions and isochromatid achromatic lesions were pooled as isochromatid lesions. One hundred metaphases per concentration were analyzed in each experiment. Data are presented as the percent metaphases with damage, which reflects the number of metaphases with at least one aberration, and as total chromosome damage, which reflects the total amount of chromosome damage in 100 metaphases.

Determination of intracellular Cr ion levels. Intracellular Cr levels in CHO cells were determined as previously described (Wise et al., 2004). Briefly, cells were seeded at a density of 500,000 cells per dish in 100-mm dishes. Cells were allowed to grow for 24 h and then treated with lead chromate for 24 h. At the end of treatment, cells were harvested immediately. Harvested cells were treated with hypotonic solution followed by 2% SDS. Solution was sheared through a needle seven times and then filtered through a 0.2-μm filter. Intracellular Cr ion levels were then measured and calculated by inductively coupled plasma mass spectrometry as previously described (Wise et al., 2004).

Data analysis. The t tests and confidence intervals for individual comparisons were calculated using Satterthwaite’s approximation for unequal variances. No adjustment for multiple comparisons was incorporated in the analysis. In order to evaluate overall differences among cell types while controlling for the effects of concentration, multiple linear regression analysis was employed (SAS Institute, 2004).

RESULTS

Intracellular Cr ion levels were measured in all three cell lines. EM9 and H9T3 cells exhibited similar levels of Cr ions, which were higher than levels found in AA8 cells treated with the same concentration (Fig. 1). Accordingly, the cytotoxicity and genotoxicity data are presented using both the administered lead chromate concentration and the intracellular Cr ion level for all three cell lines.

Lead chromate induced concentration-dependent decreases in relative survival of all three cell lines (Fig. 2A). It induced only a modest amount of cell death in AA8 cells and an increased amount in XRCC1-deficient EM9 cells. Complementing the EM9 cells with XRCC1 (H9T3 cells) restored the cytotoxic response to normal levels. This pattern remained the same when the data were corrected for differences in intracellular uptake levels (Fig. 2B). Thus, XRCC1 plays a role in preventing lead chromate–induced cytotoxicity.

Lead chromate induced concentration-dependent increases in clastogenicity in AA8, EM9, and H9T3 cells (Fig. 3). It damaged a similar percentage of metaphases in all three cell lines. Specifically, 0.1, 0.5, and 1 μg/cm² damaged 10, 19, and 20 metaphases in AA8; 14, 22, and 25 metaphases in EM9; and...
9, 17, and 20 metaphases in H9T3 cells, respectively (Fig. 3A). A similar pattern was seen after correcting for differential uptake (Fig. 3B). Thus, XRCC1 deficiency does not appear to affect the frequency of a metaphase incurring chromosome damage after lead chromate exposure.

Lead chromate also induced concentration-dependent increases in total chromosome damage (Fig. 4). Total chromosome damage represents total frequencies of aberrations per 100 cells. There was a consistent increase in the amount of total chromosome damage in EM9 cells compared to AA8 cells, and complementing the cells with XRCC1 restored the wild-type phenotype (Fig. 4A). Specifically, lead chromate concentrations of 0.1, 0.5, and 1 μg/cm² induced 12, 22, and 25 chromosome aberrations in AA8 cells; 25, 49, and 52 in EM9; and 9, 20, and 30 in H9T3 cells, respectively (Fig. 4A). Adjusting for different uptake levels showed an even stronger effect for XRCC1 deficiency (Fig. 4B). Thus, XRCC1 protects cells from total particulate Cr(VI)-induced chromosome damage.

We inspected the spectrum of chromosome damage to determine which types of lesions were most affected by XRCC1 deficiency. Chromatid lesions were the most frequently induced lesions in all three cell lines (Fig. 5). The absence of XRCC1 (EM9 cells) increased the number of chromatid lesions, and complementing the cells with XRCC1 (H9T3 cells) restored the damage levels to those observed in wild-type cells (AA8). By contrast, levels of isochromatid lesions were similar in all three cell lines and were not affected by XRCC1 deficiency (Figs 6A and 6B). Chromatid exchanges were much less frequent in all three cell lines but were much more prevalent in XRCC1-deficient EM9 cells compared to the wild-type AA8 and the XRCC1-complemented H9T3 cells (Fig. 7). Thus, XRCC1 protects cells from lead chromate–induced chromatid lesions and exchanges but does not appear to have a role in preventing isochromatid lesions.

DISCUSSION

Cr(VI)-induced tumors exhibit CIN, but the mechanisms are unknown (Hirose et al., 2002). Particulate Cr(VI) compounds are more potent lung carcinogens than soluble ones. These compounds are complex genotoxicants inducing a spectrum of DNA damage, but the mechanisms for repairing these lesions are uncertain and their relationship to CIN is unknown. In this study we show that XRCC1 is necessary for protecting cells from lead chromate–induced chromosome damage. Inefficient repair in XRCC1-deficient cells leads to more complex damage and CIN. Our data indicate that XRCC1 deficiency does not affect the frequency of cells that incur Cr(VI)-induced damage, but it alters the total amount of damage that occurs within a cell that does incur such damage. These data are the first to consider genes involved in repair
of particulate Cr(VI)-induced chromosome damage. They are consistent with previous reports of functional polymorphisms in XRCC1, which correlate with increased chromosome damage in exposed workers (Lei et al., 2002; Mateuca et al., 2005).

XRCC1 interacts with a number of proteins involved in SSB break repair, serving as a scaffold connecting many of the other proteins involved in SSB repair such as PCNA, POLβ, and PNK (Caldecott, 2003; Fan et al., 2004; Kubota et al., 1996; Vidal et al., 2001; Whitehouse et al., 2001). Thus, our data strongly suggest that DNA SSBs are important lesions that underlie chromatin lesions. This conclusion is consistent with previous reports indicating that soluble Cr(VI)-induced SSB correlate positively with Cr(VI)-induced chromosome damage (Bianchi et al., 1980; Levis and Majone, 1979; Sugiyama et al., 1993). Our data also indicate that failure to repair these SSBs leads to more complex chromosome damage such as chromatid exchanges. Generation of chromatid exchanges may lead to an increase in chromosome translocations, a hallmark of most cancers (Cleary, 1991; Croce, 1986; Testa, 1990).

We also found that XRCC1-deficient cells are more sensitive to the cytotoxic effects of particulate Cr(VI). These data suggest that XRCC1 may also be involved in the pathway for cell survival in cells exposed to lead chromate. These observations are different than those observed for soluble Cr(VI) and other metals, which found that EM9 cells were not more sensitive to the cytotoxic effect of these compounds (Christie et al., 1984; Thompson and West, 2000). One possible explanation for this difference may be the fact that lead chromate is a relatively insoluble Cr(VI) compound, and some of the particles are internalized by phagocytosis (Wise et al., 1993; Xie...
et al., 2004). Indeed, a previous study in human lung cells found that the majority, but not all, of the cytotoxicity induced by lead chromate was the result of partial extracellular dissolution of the particles generating extracellular soluble Cr(VI) ions; however, some of lead chromate’s cytotoxicity was not related to chromium but rather to the internalization of the particles (Holmes et al., 2005; Xie et al., 2004).

Our data also show that cells deficient in DNA repair may have dramatically different intracellular levels of chemicals from their parent cells (Fig. 1). These observations are important because, while previous DNA repair studies have tended to focus on radiation-induced effects, which do not require cellular uptake, recent studies are beginning to consider the repair of lesions induced by chemicals such as mitomycin C and psoralen. It is essential that these studies determine intracellular levels to ensure that differences observed in parent and DNA repair–deficient cells actually reflect deficiencies in repair and not simply differences in chemical uptake, particularly in instances when a single dose is considered.

EM9 cells have been in culture for more than 25 years (Caldecott, 2003). It is certainly possible that there are factors in addition to XRCC1 that could play a factor in the greater sensitivity of these cells to the cytotoxic and genotoxic effects of particulate Cr(VI). For these endpoints, other factors such as metabolic differences or defects in other repair pathways may play a role. However, the fact that adjusting for differences in Cr uptake and complementing the cells with XRCC1 did correct the sensitivity with respect to total chromosome damage (Fig. 4B), chromatid lesions (Fig. 5B), and chromatid exchanges (Fig. 7B) does indicate that XRCC1 is the key factor for these events.

In summary, we have shown for the first time that XRCC1 is involved in protecting cells from particulate Cr(VI)-induced CIN. Taken together, these results begin to describe an
XRCC1 protects against particulate chromate–induced chromosome damage and cytotoxicity

FIG. 7. Chromatid exchanges induced in AA8, EM9, and H9T3 cells after the 24-h treatment with lead chromate. Significant levels of chromatid exchange occur only when cells are deficient in XRCC1 (p < 0.05). XRCC1 protects cells from lead chromate–induced chromatid exchanges. There were no chromatid exchanges detected in control cells. Control data for each cell line in each experiment were subtracted from results obtained for each treatment concentration. Data represent mean of at least three independent experiments ± SEM. (A) Data based on administered concentration after the 24-h treatment. (B) Data based on intracellular Cr ion levels after the 24-h treatment show a significant increase in chromatid exchanges in EM9 cells compared to AA8 and H9T3 cells (p < 0.05).

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<thead>
<tr>
<th>Chromatid Exchanges</th>
<th>Lead Chromate Concentration (µg/cm²)</th>
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<tbody>
<tr>
<td>AA8-24h</td>
<td>0.1</td>
</tr>
<tr>
<td>EM9-24h</td>
<td>0.5</td>
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<tr>
<td>H9T3-24h</td>
<td>1</td>
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We would like to thank Larry Thompson for the generous gift of the AA8, EM9, and H9T3 cells. This work was supported by National Institute of Environmental Health Sciences grant ES10838 (J.P.W.) and the Maine Center for Toxicology and Environmental Health at the University of Southern Maine.

ACKNOWLEDGMENTS


Matutea, R., Aka, P. V., De Boeck, M., Hauspie, R., Kirsch-Volders, M., and Lison, D. (2005). Influence of hOGG1, XRCC1 and XRCC3 genotypes on explanation for the initial mechanism for particulate Cr(VI)-induced CIN. Specifically, Cr(VI) particles partially dissolve outside cells generating Cr(VI) ions that enter cells via an anion transport system (DeFlora et al., 1989; Wetterhahn and Hamilton, 1989). Once inside the cell, Cr(VI) is reduced through a complex metabolic pathway to Cr(III) by reducing agents like vitamin C or glutathione (DeFlora et al., 1989; Wetterhahn and Hamilton, 1989). Intermediate Cr species, including Cr(V), Cr(IV), and reactive oxygen species, are generated after intracellular reduction of Cr(VI). These Cr intermediates and reactive oxygen species are the key factors for DNA SSB (Xu et al., 1992). These DNA SSB are repaired by pathways involving XRCC1 with unrepaired breaks leading to chromatid lesions and exchanges and potentially translocations.


