Ku80 Deficiency Does Not Affect Particulate Chromate–Induced Chromosome Damage and Cytotoxicity in Chinese Hamster Ovary Cells

Eric Camyre,* Sandra S. Wise,* Peter Milligan,+ Nancy Gordon,* Britton Goodale,* Megan Stackpole,* Natalie Patzlaff,* AbouEl-Makarim Aboueissa,‡ and John Pierce Wise, Sr*†

*Wise Laboratory of Environmental and Genetic Toxicology, Maine Center for Toxicology and Environmental Health, University of Southern Maine, Portland, Maine 04104-9300; †College of Natural and Social Sciences, University of Maine at Augusta, Augusta, Maine 04330-9410; and ‡Department of Mathematics and Statistics, University of Southern Maine, Portland, Maine 04104-9300

Received January 18, 2007; accepted February 18, 2007

Particulate hexavalent chromium (Cr(VI)) compounds are human lung carcinogens. These compounds induce DNA damage, chromosome aberrations, and concentration-dependent cell death in human and Chinese hamster ovary (CHO) cells. The relationship between Cr(VI)-induced DNA damage and chromosome aberrations is poorly understood. Accordingly, this study focused on examining the role of Ku80, a gene involved in nonhomologous end-joining repair, in particulate chromate-induced cytotoxicity and chromosome damage in CHO cells. Three different cell lines were used: CHO-K1 (parental), xrs-6 (Ku80 deficient), and 2E (xrs-6 complemented with Ku80 gene). Levels of cell death were higher in xrs-6 cells when compared to wild type, suggesting that Ku80 was important for protecting cells from lead chromate. However, Ku80 played no role in protecting cells from particulate Cr(VI)-induced chromosome instability (CIN) as gene complementation with Ku80 (2E cells) studies and uptake experiments showed similar frequency and amounts of chromosome damage between the cell lines and that any observed difference based on administered concentration was actually due to differences in Cr(VI) uptake. The spectrum of chromosome damage was also unaffected by Ku80 deficiency. These data indicate that Ku80 protects cells from cytotoxicity but is not involved in protecting cells from particulate chromate-induced CIN.

Key Words: chromium; chromate; particulate; Ku80; chromosome instability; single-strand breaks; nonhomologous end-joining repair.

Hexavalent chromium (Cr(VI)) compounds are established human lung carcinogens. Chromosome instability (CIN) is a hallmark of lung cancer, and recent data extend these observations to include Cr(VI)-induced tumors (Hirose et al., 2002; Masuda and Takahashi, 2002; Nakamura et al., 2003; Takahashi et al., 2005). More specifically, epidemiological and toxicological studies indicate that the potent form of carcinogenic Cr(VI) include the water insoluble or “particulate” compounds. These compounds induce tumors in experimental animals and neoplastic transformation of cultured cells (IARC, 1990; Levy et al., 1986; Patierno et al., 1988). The genotoxic mechanism for these Cr(VI) particles involves extracellular dissolution releasing the cation and the chromate anion, which both enter the cell (Wise et al., 2004b). Once inside the cells, the chromate anions induce DNA and chromosome damage, growth arrest, and cytotoxicity, while the cation has no effect (Singh et al., 1999; Wise et al., 1993, 2002, 2004b; Xu et al., 1992). Particulate Cr(VI) compounds induce CIN manifested as structural chromosomal aberrations (Wise et al., 1992), but the mechanisms involved in these effects are unknown.

Lead chromate, a model particulate Cr(VI) compound, is a potent clastogen inducing chromosomal aberrations in different species and cell types (DeFlora et al., 1990; Wise et al., 1992, 2002). Previously, it has been shown that other particulate Cr(VI) compounds exhibit a similar cytotoxic and genotoxic response as lead chromate (Wise et al., 2004c). This clastogenic effect is mediated by soluble extracellular Cr(VI) ions while the lead ions are not involved in the cytotoxicity or genotoxicity (Holmes et al., 2005; Xie et al., 2004, 2005). Lead chromate also induces DNA double-strand breaks, which correlate with chromosome damage (Xie et al., 2004). Based on these data, we hypothesize that efficient repair of particulate Cr(VI)-induced DNA double-strand breaks is necessary to protect cells from Cr(VI)-induced chromosome damage and CIN.

DNA double-strand breaks are repaired through two systems, nonhomologous end-joining (NHEJ) and homologous recombination (HR) repair (Jackson, 2002). NHEJ essentially ligates the end of the break together and occurs throughout the cell cycle, while HR repair copies the missing information from a sister chromatid and is restricted to S and G2 after a sister chromatid has been synthesized (Jackson, 2002). NHEJ
**Ku80 DEFICIENCY AND Cr(VI)-INDUCED CHROMOSOME DAMAGE AND CYTOTOXICITY IN CHO CELLS**

Ku80 is thought to be the dominant repair pathway for preventing CIN in mammalian cells (Weinstock et al., 2006); however, it is currently unknown if it serves to prevent Cr(VI)-induced CIN.

Ku80 is a gene involved in NHEJ repair (Jackson, 2002). It forms a heterodimer with Ku70 and together they serve as a sensor of theBreaks and hold the ends together (Jackson, 2002). It is unknown if Ku80 is involved in preventing Cr(VI)-induced CIN. Thus, the purpose of our study was to examine the importance of NHEJ in preventing particulate chromate–induced CIN by examining the impact of Ku80 deficiency on the amount and spectrum of particulate Cr(VI)–induced chromosome damage.

**MATERIALS AND METHODS**

**Reagents and chemicals.** Lead chromate, colchicine, and potassium chloride were purchased from Sigma-Aldrich (St Louis, MO). Dulbecco’s minimal essential medium with Ham’s F-12 medium (DMEM/F12) was purchased from Mediatech (Herndon, VA). Giemsa stain was purchased from Biomedical Specialties Inc. (Santa Monica, CA). Cosmic calf serum was purchased from HyClone (Logan, UT). Sodium pyruvate, penicillin/streptomycin, Gluta-MAX, phosphate buffered saline 1× (PBS), and trypsin-EDTA were purchased from Invitrogen Corporation (Grand Island, NY). Acetone, crystal violet, and methanol were purchased from J.T. Baker (Phillipsburg, NJ). Tissue culture dishes, flasks, and plasticware were purchased from Corning Inc. (Acton, MA).

**Cell lines and cell culture.** CHO-K1 serve as the wild-type control and were a gift of Larry Thompson of Lawrence Livermore National Laboratory. The xrs-6 cells were originally derived from CHO-K1 cells and are known to be Ku-80 deficient (−), specific to NHEJ (Jeggo and Kemp, 1983). 2E cells are Ku80-cDNA–complemented xrs-6 cells, which reverses the repair deficiency seen in xrs-6 cells (Singleton et al., 1997). Levels of Ku-80 in the wild type (K1) and the complement (2E) have been previously shown to be similar (Singleton et al., 1997). The xrs-6 cells and 2E cells were a gift from Penny Jeggo of the University of Sussex. All cell lines were cultured in 50:50 DMEM/F12 medium 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, fed every second day, and subcultured at least twice a week.

**Lead chromate preparation.** Lead chromate (CAS # 7758-97-6, ACS reagent minimum 98% purity) was used as a model for the particulate form of Cr(VI) and was administered as a suspension in acetone as previously described (Wise et al., 2002). Briefly, suspensions of PbCrO4 particles were prepared by rinsing twice in double-distilled water to remove any water-soluble contaminants and then twice in acetone to remove any organic contaminants. Air-dried particles were weighed, placed in acetone (for sterilization) in a borosilicate scintillation vial, and stirred overnight with a magnetic stir bar. Next, the particles were kept in suspension using a vortex mixer and diluted into appropriate suspension for specific treatments. Dilutions were also maintained as a suspension using a vortex mixer, and treatments were dispensed into cultures directly from these suspensions. Acetone concentrations were kept at 0.5% or below which were nontoxic (data not shown).

**Cytotoxicity assay.** Cytotoxicity was determined with a clonogenic assay measuring reduction in plating efficiency of treatment groups relative to controls as previously described (Wise et al., 2002). Briefly, 90,000 cells were seeded in each well of six-well dishes and allowed to grow for 24 h so that cells could resume log phase growth. Cells were then treated for 24 h with lead chromate. At the end of treatment, cells were rinsed with PBS, trypsinized with 0.25% trypsin-EDTA, and reseeded at a density of 200 cells per dish in each of four 60-mm dishes. After 7 days, colonies were stained with crystal violet and counted. All experiments were performed at least three times with four dishes per treatment group.

**Clastogenicity.** Clastogenicity was measured as the amount of chromosome damage in treatment groups and controls exactly as previously described (Wise et al., 2002). Cells were treated for 24 h. Each experiment was repeated three times.

**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 scored as “lesions” as previously described (Wise et al., 1992). This was done because breaks can only be unequivocally distinguished from gaps lesions if the distal acentric 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 chromatin. Accordingly, chromatid deletions and achromatic lesions were pooled as chromatid lesions while isochromatid deletions and isochromatid acentric 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. Additionally, a mitotic index was performed without adding colchicine. There was no difference in the frequency of mitotic cells between the three cell lines after lead chromate exposure (data not shown).

**Determination of intracellular Cr ion levels.** The intracellular concentrations of the metals were determined as previously described (Holmes et al., 2005). Briefly, a monolayer of cells was treated with lead chromate for 0 and 24 h. Cells were then harvested and placed in a hypotonic solution followed by 2% SDS to degrade the cell membrane. This solution was then sheered through a needle seven times and filtered in order to remove remaining undissolved lead chromate particles. Cr and/or Pb ion concentrations of the samples were then measured by inductively coupled plasma atomic emission spectrometry (ICP-AES) as described previously (Holmes et al., 2005). Zero-hour harvests (i.e., cells were treated and immediately harvested within moments) were performed with lead chromate to account for the possibility that some undissolved lead chromate particles passed through the filter. The final concentrations were corrected for this possible confounding factor by subtracting 0-h values from the 24-h values as previously described (Holmes et al., 2005).

**Data analysis.** Parametric and nonparametric ANOVAs were used to determine whether there was evidence of differences among the K1 (wild type), xrs-6 (Ku80), and 2E (complemented Ku80) for different lead chromate concentration levels (0.1, 0.5, 1.0, 5.0, 10.0 µg/cm²). The t-test and the nonparametric Mann-Whitney test were used to compare the results between each of the cell lines for each treatment concentration.

**RESULTS**

**Uptake**

Intracellular Cr ion levels were measured in all three cell lines. The xrs-6 and 2E cells exhibited similar levels of Cr ions, which were higher than levels found in CHO-K1 cells treated with the same concentration (Fig. 1). Accordingly, the cytotoxicity and genotoxicity data are presented using both administered lead chromate concentration and intracellular Cr ion level for all three cell lines.

**Cytotoxicity**

There was a concentration-dependent decrease in relative survival for each of the three cell lines after lead chromate...
exposure (Fig. 2A). The xrs-6 Ku80-deficient cell line was more susceptible to lead chromate compared to the K1 parental cell line, particularly at higher concentrations. For example, at 1 µg/cm², 8% relative survival was seen in the xrs-6 cell line, compared to 78% relative survival in the parental K1. Complementing the xrs-6 cells with Ku80 (2E 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), indicating that Ku80 plays a role in preventing lead chromate–induced cytotoxicity.

Chromosome Damage

For all three cell lines, there was a concentration-dependent increase in the percentage of chromosome damage (Fig. 3A). At low concentrations, there was no difference between the three cell lines, but at the highest concentration, the wild type showed less damage than the Ku80(−) cells. However, both the Ku80(−) and Ku80-complemented cells exhibited similar frequencies of damaged metaphases, suggesting that the difference between the Ku80(−) and wild-type cells was not related to Ku80. Correcting for differences in intracellular uptake showed little difference between the cell lines (Fig. 3B), confirming that Ku80 deficiency does not appear to affect the frequency of a metaphase incurring chromosome damage after lead chromate exposure.

The total amount of chromosome damage also increased in a concentration-dependent manner (Fig. 4). At low concentrations, there was again no difference among the three cell lines. At the highest concentration, the wild type again showed less damage than the Ku80(−) cells. However, both the Ku80(−) and Ku80-complemented cells also exhibited a similar amount of total damage, suggesting that the difference between the Ku80(−) and wild-type cells was not related to Ku80. Correcting for differences in intracellular uptake showed little difference among the cell lines (Fig. 4B), thus confirming that Ku80 deficiency does not appear to affect the amount of chromosome damage after lead chromate exposure.

We also considered the spectrum of damage in each cell line. Figure 5 shows that the most common aberrations observed in the three cell lines exposed to lead chromate were chromatid lesions and that Ku80 deficiency did not change the spectrum of damage. These data further support the conclusion that Ku80 is not affecting particulate Cr(VI)–induced chromosome damage.

DISCUSSION

CIN is common in lung cancer and 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 compounds. They are complex genotoxicants inducing a spectrum of DNA damage, including DNA strand breaks, Cr-DNA adducts, and Cr-DNA cross-links, but the mechanisms for repairing these lesions are uncertain.
Ku80 does not play a role in protecting cells from lead chromate–induced chromosome damage as we did not find a change in the amount or type of chromosome damage. This conclusion is consistent with previous data that show that Cr(VI)-induced DNA double-strand breaks are preferentially formed after the S-phase of the cell cycle possibly from replication fork collapse resulting from the repair of a single-strand break, cross-link, or adduct and do not form in G1 (Ha et al., 2004) and observations that NHEJ predominates in G1 with HR repair dominating in S and G2 (Ferreira and Cooper, 2004; van den Bosch et al., 2002). Any unrepaired double-strand breaks then manifest as chromatid aberrations in mitosis. The proximate genotoxicant for particulate and soluble Cr(VI) is the same (chromate anion), and thus, the mechanism of formation of the double-strand break should also be the same (Xie et al., 2004). We have also reported preliminary data that HR repair does protect cells from particulate Cr(VI)–induced CIN (Wise et al., 2006). These data are the first to consider genes involved in NHEJ repair and Cr(VI)-induced chromosome damage.
We found that Ku80-deficient cells are more sensitive to the cytotoxic effects of particulate Cr(VI). These data are consistent with reports that show cells deficient in XRCC1 and FANCG are also sensitive to the cytotoxic effects of particulate Cr(VI) (Grlickova-Duzevick et al., 2006a; Savery et al., 2007). However, unlike in those reports, here there was no effect on CIN. Considered together, these reports suggest that there is an interaction between the repair pathway and the cell death pathway for particulate Cr(VI) that is not the result of an increase in chromosomal damage. It is not readily clear why we see more cell death without any increase in chromosome damage as there is little data considering the role of Ku80 in the absence of DNA damage. Our data suggest that Ku80 may have some role in sensing DNA damage and preventing the cells from undergoing apoptosis while repair is completed. Some data suggest that this role may occur. For example, expression of a dominant-negative mutant Ku increases apoptosis in pancreatic acinar cells, which is consistent with our data (Song et al., 2003). By contrast, overexpression of Ku80 increases radiation-induced apoptosis (Chang et al., 2006).

We found that Ku80-deficient cells do not affect the frequency, amount, or spectrum of CIN induced by particulate Cr(VI), but the data are consistent with levels of these lesions observed in human lung cells treated for 24 h (Wise et al., 2004a). These data differ from our previous reports of XRCC1 and FANCG deficiency. Previously, we showed that XRCC1 protects cells from lead chromate–induced chromosome damage (Grlickova-Duzevick et al., 2006a). XRCC1 is known to interact with a number of proteins involved in SSB break repair, serving as a scaffold to connect them (Caldecott, 2003). More recently, XRCC1 has been shown to be involved in DNA double-strand break repair. XRCC1 deficiency increased Cr(VI)-induced CIN, manifested as a greater amount of chromosome damage and as more complex chromosome aberrations 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).

FANCG-deficient cells also exhibited an increase in the amount of particulate Cr(VI)–induced CIN, but FANCG deficiency did not alter the spectrum of Cr(VI)–induced chromosomal damage. Thus, considering these two studies with the present study indicates that NHEJ repair is not involved in protecting cells from CIN but that a strand break repair pathway is involved. More than likely, this pathway is the HR repair pathway as Cr(VI) has recently been shown to induce HR repair (Bryant et al., 2006) and single-strand breaks do not correlate well with chromosome damage as they are rapidly repaired within hours of Cr(VI) exposure (Xu et al., 1992) while Cr(VI)-induced chromosome damage persists for days (Holmes et al., 2006).

Our data also show that DNA repair–deficient cells may have dramatically different intracellular levels of chemicals from their parent cells (Fig. 4). The observations are consistent with our previous reports of XRCC1- and FANCG-deficient cells, which also found large differences between parent cells and the repair-deficient cells derived from them (Grlickova-Duzevick et al., 2006a,b; Savery et al., 2007). Correcting for differences in uptake can reverse conclusions made based on administered dose alone. These observations are important because recent studies are beginning to consider the repair of lesions induced by chemicals such as mitomycin C, psoralen, and Cr(VI) but are failing to account for uptake differences. For example, a recent paper about Cr(VI)-induced HR repair compared HR repair–deficient cells to the parent cells and reported a significant difference, but the fact that the observed differences could have been caused by differential uptake was not considered or evaluated (Bryant et al., 2006). Thus, it is uncertain if the differences observed in that study were due to the genes under study or a difference in Cr(VI) uptake.

In summary, we have shown for the first time that Ku80 is not involved in protecting cells from particulate Cr(VI)–induced CIN, and thus, NHEJ appears to be a minor pathway for repair of Cr(VI)-induced DNA double-strand breaks. This finding is consistent with observations that Cr(VI)-induced DNA double-strand breaks do not form in G1 when NHEJ is the major double-strand break repair pathway. Further work will focus on the importance of the HR repair pathway in protecting cells from particulate Cr(VI)–induced CIN.
ACKNOWLEDGMENTS

We would like to thank Larry Thompson for the generous gift of the CHO-K1 cells. We would also like to thank Penny Jeggo for the generous gift of the xrs-6 and E2 cells. We would like to thank David Kirstein for administrative support and Christy Gianios for information technology support. We also would like to thank Dr. Samantha Langley-Turnbaugh for the use of the ICP-AES. 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.

REFERENCES


