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Chronic Exposure to Zinc Chromate Induces Centrosome Amplification and Spindle Assembly Checkpoint Bypass in Human Lung Fibroblasts

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Abstract

Hexavalent chromium (Cr(VI)) compounds are known human lung carcinogens. Solubility plays an important role in its carcinogenicity with the particulate or insoluble form being the most potent. Of the particulate Cr(VI) compounds, zinc chromate appears to be the most potent carcinogen, however, very few studies have investigated its carcinogenic mechanism. In this study, we investigated the ability of chronic exposure to zinc chromate to induce numerical chromosome instability. We found no increase in aneuploidy after a 24 hour exposure to zinc chromate, but with more chronic exposures, zinc chromate induced concentration- and time-dependent increases in aneuploidy in the form of hypodiploidy, hyperdiploidy and tetraploidy. Zinc chromate also induced centrosome amplification in a concentration- and time-dependent manner in both interphase and mitotic cells after chronic exposure, producing cells with centriolar defects. Further, chronic exposure to zinc chromate induced concentration- and time-dependent increases in spindle assembly checkpoint bypass with increases in centromere spreading, premature centromere division and premature anaphase. Lastly, we found that chronic exposure to zinc chromate induced a G2 arrest. All together, these data indicate that zinc chromate can induce chromosome instability after prolonged exposures.

Keywords

chromium; zinc chromate; numerical chromosome instability; aneuploidy; centrosome amplification; spindle assembly checkpoint

Introduction

Hexavalent chromium compounds (Cr(VI)) are well-established human lung carcinogens with the water insoluble ('particulate') form being the most potent (1–4). Human pathology studies

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indicate that inhaled particles impact and persist in the lung bifurcations sites where Cr-induced tumors occur and these data are consistent with a particulate exposure (5–6). In general, the carcinogenic mechanism for chromate compounds is poorly understood (*reviewed in 7*). Current data suggest that a multistage carcinogenesis model, which requires the stepwise acquisition of mutations in key genes does not fit chromate carcinogenesis well, as Cr(VI) is a very weak mutagen and thus, is unlikely to induce sufficient mutations for this mechanism (*reviewed in 7*). A more probable mechanism for chromate carcinogenesis is genomic instability, which requires disruption of the control of genomic stability due to interference with DNA repair, kinetochore assembly, checkpoints, centrosome duplication, microtubule dynamics and numerous other cellular maintenance processes (*reviewed in 7*).

Genomic instability can manifest itself as microsatellite instability (MIN) and/or chromosome instability (CIN). Lung tumors are known to simultaneously exhibit both MIN and CIN (8). CIN instability can consist of either numerical or structural chromosomal abnormalities. Data show that lung tumors in occupationally exposed Cr(VI) workers exhibit MIN, though studies of CIN have not yet been performed in these tumors (9,10). Currently, no cell culture studies have shown that Cr(VI) can cause MIN or disrupt the mismatch repair genes thought to underlie the MIN. However, studies in cells deficient in mismatch repair genes show decreased double strand break and micronuclei formation (11,12) suggesting that mismatch is important in protecting cells against Cr(VI).

Considering CIN, studies in humans, animal and cell culture all show that both particulate and soluble Cr(VI) compounds can induce chromosomal aberrations, which suggest the ability to induce structural chromosomal changes (9,13–19). Interestingly, no studies have reported translocations after Cr(VI) exposure. Cell culture studies also show that lead chromate can induce numerical CIN in lung cells (20–21). These data are consistent with lung tumors in general, which typically exhibit an aneuploid phenotype (22). The underlying mechanism for lead chromate-induced numerical CIN involved disruption of the spindle assembly checkpoint (23) and an increase in centrosome number (24–25), though effects on centrioles were not considered (20). Studies with other particulate Cr(VI) compounds have not been done so it is uncertain if these effects are specific to lead chromate or more generalizable to other particulate Cr(VI) compounds. Thus, it is unclear if the genomic instability model reflects a general response to Cr(VI) compounds or is limited to this specific compound.

Insoluble chromium compounds used in chromate pigment production include lead chromate, zinc chromate, barium chromate and strontium chromate with lead chromate and zinc chromate being the most widely used compounds (19). Of the particulate compounds, zinc chromate appears to be the most potent carcinogen (26–30). Numerous epidemiology studies specifically link zinc chromate exposure to an increased risk of lung cancer (26–30). Langard and Vigander found the observed/expected ratio for lung cancer in their chromate-exposed cohort was 44 and all but one of their cases was specifically exposed to zinc chromate (29). Davies, 1984 reported similar findings with workers exposed to zinc chromate experiencing increased cancer risk, but workers at chromate production plants that produced only lead chromate exhibited normal mortality rates (27). Whole animal studies confirm the epidemiological studies and show that, of 20 chromium compounds tested, zinc chromate was one of only three chromium compounds that significantly increased bronchial carcinomas in the rat lung (31).

However, despite its potent carcinogenicity, few studies have investigated the mechanism of zinc chromate carcinogenesis. An early cell culture study performed in the 1980s showed that zinc chromate can transform Syrian hamster cells, confirming its carcinogenic potential in culture (32). Only two other cell culture studies have been performed since that early study. One study investigated oxidative stress and found that zinc chromate exposure lead to oxidation of thioredoxin 1 and 2 (33). The other study investigated the genotoxicity of zinc chromate and

found that a 24 h exposure induced chromosome aberrations and DNA double strand breaks in human lung cells (34). No study has addressed the ability of this important carcinogen, zinc chromate, to induce numerical chromosome instability, disrupt the spindle assembly checkpoint or alter centrosome number, which are key elements in the CIN aspects of the genomic instability model. Accordingly, the present study investigates the effects of zinc chromate, on the spindle assembly checkpoint and on chromosome and centrosome number in human lung cells. In addition, the study further explores this mechanism by reporting effects on centrioles within the centrosomes, which previously has not been considered for any metal.

Materials and Methods

Reagents

Zinc chromate, demecolchicine, potassium chloride, magnesium sulfate, EGTA, PIPES, anti- γ -tubulin (clone GTU-88), anti- α -tubulin-FITC conjugate antibody and Triton X-100 were purchased from Sigma/Aldrich (St. Louis, MO). Giemsa stain was purchased from Biomedical Specialties Inc. (Santa Monica, CA). Alexa Fluor 555 goat anti-mouse IgG, propidium iodide and Prolong with DAPI were purchased from Molecular Probes (Eugene, OR). Anti-centrin was provided as a gift from Dr. Jeffery Salisbury from the Mayo Clinic in Rochester, MN. DyLight 549-conjugated goat anti-mouse IgG1 and DyLight 488-conjugated goat anti-mouse IgG2a were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Trypsin/EDTA, sodium pyruvate, penicillin/streptomycin, Glutamax and Gurr's buffer were purchased from Invitrogen Corporation (Grand Island, NY). Methanol, acetone and acetic acid were purchased from J.T. Baker (Phillipsburg, NJ). Dulbecco's minimum essential medium and Ham's F-12 (DMEM/F-12) 50:50 mixture was purchased from Mediatech Inc. (Herndon, VA). Cosmic calf serum (CCS) was purchased from Hyclone (Logan, UT). Tissue culture dishes, flasks and plasticware were purchased from Corning Inc. (Acton, MA).

Cells and Cell Culture

WTHBF-6 cells were used in all experiments. WTHBF-6 cells, a clonal cell line derived from normal human bronchial fibroblasts, ectopically express human telomerase. These cells have been grown for over 2000 population doublings and continue to exhibit a normal stable karyotype. After metal exposure, these cells exhibit similar clastogenic and cytotoxic responses compared to their parent cells (16). Cells were maintained as subconfluent monolayers in DMEM/F-12 supplemented with 15% CCS, 2 mM glutamax, 100 U/ml penicillin/100 ug/ml streptomycin and 0.1 mM sodium pyruvate and incubated in a 5% CO₂ humidified environment at 37°C. They were fed three times a week and sub-cultured at least once a week using 0.25% trypsin/1mM EDTA solution. All experiments were performed on logarithmically growing cells.

Metal Preparation

Zinc chromate was administered as a suspension in cold water, as previously described (34). Cells were treated with 0, 0.1, 0.15 and 0.2 ug/cm² zinc chromate for 24, 48, 72, 96 and 120 h.

Cytotoxicity

Cytotoxicity was measured with a clonogenic survival assay using standard methods (14). Clonogenic survival assays measure the reduction in plating efficiency in treatment groups relative to control dishes. Each treatment group contained four dishes and each independent experiment was repeated at least three times. The plating efficiency was consistently between 10–15% for controls.

Aneuploid Analysis

Aneuploidy was determined by counting the number of chromosomes in solid stained metaphases. Chromosome preparation was performed as previously described (14). Each experiment was repeated at least three times. A minimum of 100 metaphases were analyzed per concentration and at least three independent experiments were performed.

Spindle Assembly Checkpoint Bypass

Spindle assembly checkpoint bypass was analyzed as previously described (21). Briefly, one hour before the end of treatment, cells were incubated with 0.1 $\mu\text{g/ml}$ demecolchicine. Cells were then harvested and incubated in 0.075M KCl for 17 minutes. Following hypotonic, cells were fixed with 3:1 methanol:acetic acid for 20 minutes and the fix was changed 2 times. Cells were dropped onto cleaned wet slides, dried and stained with Giemsa. Metaphases were analyzed for centromere spreading, premature centromere division and premature anaphase. At least 100 metaphases per concentration and time point were analyzed and at least three independent experiments were performed.

Centrosome and Microtubule Analysis

Centrosome and microtubule analysis was performed as previously described (20). Briefly, cells were seeded onto glass chamber slides and treated with 0, 0.1, 0.15 or 0.2 $\mu\text{g/cm}^2$ zinc chromate for 24, 48, 72, 96 or 120 h. Cells were rinsed 2x in a microtubule stabilizing buffer (3 mM EGTA, 50 mM PIPES, 1 mM magnesium sulfate, 25 mM potassium chloride), fixed with -20°C methanol for 10 minutes and rehydrated with 0.05% Triton X-100 for 3 minutes. Blocking buffer was then added to the cells for 30 minutes followed by one hour incubation with a primary anti- γ -tubulin antibody (Sigma, T-6557). Cells were washed four times with PBS and then incubated with Alexa Fluor 555 goat anti-mouse IgG secondary antibody for an hour in the dark. After the hour incubation, cells were washed and then incubated with anti- α -tubulin-FITC conjugated antibody for one hour in the dark followed by four PBS washes. Cells were washed with water and coverslips were mounted with Prolong anti-fade with DAPI. One hundred mitotic cells and 1000 interphase cells were analyzed per concentration using fluorescence microscopy. At least three independent experiments were performed.

Centrin Analysis

Cells were seeded onto glass chamber slides and treated with 0 or 0.2 $\mu\text{g/cm}^2$ zinc chromate for 24, 48, 72, 96 or 120 h. After treatment, cells were washed twice with a microtubule stabilizing buffer described above and fixed with -20°C methanol for 10 minutes. Cells were then rehydrated with 0.05% Triton X-100 for 3 minutes followed by blocking buffer for 30 minutes. Cells were incubated with anti-centrin and anti- γ -tubulin for one hour, washed four times with 0.05% Triton X-100 and then incubated with DyLight 549-conjugated goat anti-mouse IgG1 and DyLight 488-conjugated goat anti-mouse IgG2a antibodies for one hour in the dark. After the secondary antibody incubation, cells were washed four times with 0.05% Triton X-100 and once with water, then coverslips were mounted with Prolong anti-fade with DAPI. Two hundred interphase and fifty mitotic cells were analyzed per concentration using fluorescence microscopy. At least three independent experiments were performed.

Cell Cycle Analysis

Cell cycle analysis was performed according to our published methods (18). Briefly, after zinc chromate exposure, cells were harvested, washed once with PBS, and fixed with -20°C 70% ethanol. Cells were allowed to fix overnight and then digested with RNase A and stained with propidium iodide for 30 minutes. PI intensity was detected using a BD FACSCalibur flow cytometer and data was analyzed using ModFit LT 3.0 software. Four independent experiments were performed.

Mitotic Index

Mitotic index was determined as previously described (35). Briefly, after zinc chromate treatment, cells were harvested and incubated in 0.075M KCl for 10 minutes. Cells were then fixed with 3:1 methanol:acetic acid for 20 minutes. The fixative was changed twice and cells were dropped onto clean wet slides. Slides were aged overnight and stained with 5% Giemsa. Mitotic cells were counted in 3000 cells and mitotic index was expressed as a percent of control. Three independent experiments were performed.

Statistics

A one-way ANOVA was used to test for significant differences among treatments and control. Tukey post-hoc and/or Dunnett comparisons were used to compare treatment levels to the control at significance level 0.05. Regression analysis was used to detect time and concentration dependency to determine if there was a statistically significant relationship between dose and time.

Results

Chronic exposure to zinc chromate induces cytotoxicity

Chronic exposure to zinc chromate induced concentration- and time-dependent decreases in clonogenic survival (Figure 1). Exposure to 0.1, 0.15 and 0.2 $\mu\text{g}/\text{cm}^2$ zinc chromate for 24 h induced 76, 64 and 53 percent relative survival, respectively, while a 120 h exposure induced 36, 24 and 16 percent relative survival, respectively. All doses and time points were statistically different from control ($p < 0.05$) and there was a statistically significant negative relationship with time at all zinc chromate concentrations and with concentration at all time points ($p < 0.05$).

Chronic exposure to zinc chromate induces aneuploidy

Previously we found that chronic exposure to lead chromate induced aneuploidy in human lung cells (20–21). In this study our goal was to determine if aneuploidy induction was lead chromate specific or a characteristic of particulate chromate compounds. We found that chronic exposure to zinc chromate induced an increase in total aneuploid cells in a concentration- and time-dependent manner (Figure 2). Total background aneuploidy levels ranged between 8 and 13 percent, which is consistent with background aneuploidy levels in primary human lung fibroblasts (36–37). After a 24 h exposure, aneuploid cells did not increase with treatment but exposure to 0.1, 0.15 or 0.2 $\mu\text{g}/\text{cm}^2$ zinc chromate for 120 h induced 28, 40 and 44 percent aneuploid metaphases (Figure 2A). There was a statistically significant positive relationship with time at all zinc chromate concentrations and with concentration at 72, 96 and 120 h ($p < 0.05$). When the aneuploid cells were grouped by chromosome number into hypodiploid (11–45 chromosomes), hyperdiploid (>46 but <92 chromosomes) and tetraploid (92 chromosomes) metaphases, we found increases in all three groups.

Hypodiploid metaphases began to increase at the highest concentration after a 72 h exposure and all three doses exhibited increased hypodiploidy after 92 and 120 h. Background hypodiploid levels ranged between 3.5 and 9 percent, but exposure to 0.1, 0.15, and 0.2 $\mu\text{g}/\text{cm}^2$ zinc chromate for 120 h induced 12, 17 and 18 percent hypodiploid metaphases, respectively (Figure 2B). Hyperdiploid cells also increased after 120 h exposure to zinc chromate. Background hyperdiploidy levels were between 0.75 and 3 percent, but a 120 h exposure to 0.1, 0.15 or 0.2 $\mu\text{g}/\text{cm}^2$ zinc chromate induced 5.5, 8.5 and 13 percent hyperdiploid metaphases (Figure 2C). Lastly, tetraploid metaphases also exhibited a time-dependent increase in tetraploid metaphases after a 72 to 120 h exposure. WHTBF-6 cells exhibited 1.7–2.7 percent background tetraploidy rate but exposure to 0.1, 0.15 and 0.2 $\mu\text{g}/\text{cm}^2$ zinc chromate for 120 h induced 11, 14 and 14 percent tetraploid metaphases, respectively (Figure 2D).

Therefore, chronic exposure to zinc chromate induces aneuploidy in the form of hypodiploidy, hyperdiploidy, and tetraploidy.

Chronic exposure to zinc chromate induces spindle assembly checkpoint bypass

Spindle assembly checkpoint bypass is one mechanism that gives rise to aneuploidy (23) and has been shown to be induced after lead chromate exposure (21). Therefore, we investigated whether zinc chromate induced spindle assembly checkpoint bypass. Cells were analyzed for centromere spreading, premature centromere division and premature anaphase (Figure 3A). Centromere spreading was defined as the disassociation of chromatids at the centromere but not at the rest of the chromosome (Figure 3A(1)). Premature centromere division was determined when at least one chromosome was separated from its sister chromatid and at least one chromosome was connected to its sister chromatid (Figure 3A(2)). Premature anaphase was defined as all chromosomes being separated from their sister chromatids (Figure 3A(3)).

We found that spindle assembly checkpoint bypass increased in a concentration- and time-dependent manner (Figure 3B). Exposure to 0.2 $\mu\text{g}/\text{cm}^2$ zinc chromate for 120 h induced 5 percent of metaphases with spreading centromeres, 21 percent premature centromere division and 13 percent premature anaphase while the control only exhibited one percent premature centrosome division and one percent premature anaphase (Figure 3B). There was a statistically significant increase in total spindle assembly checkpoint bypass with time at all zinc chromate concentrations and with concentration at 96 and 120 h ($p < 0.05$).

Chronic exposure to zinc chromate induces centrosome amplification

Centrosome amplification is known to cause aneuploidy via multipolar spindle formation and division (24–25). Therefore, we investigated whether zinc chromate caused centrosome amplification, considering both interphase and mitotic cells after chronic exposure. We found that zinc chromate induced centrosome amplification in a concentration and time-dependent manner in interphase and mitotic cells (Figures 4–5). Normal interphase cells have either one or two centrosomes depending on the phase of the cell cycle (Figure 4A(1–4)) and abnormal interphase cells have more than two centrosomes (Figure 4A(5–8)). The percentage of interphase cells with greater than two centrosomes increased in a concentration- and time-dependent manner. Background interphase centrosome amplification ranged between 2.5 and 4.9%. After 24h, we observed no increase in centrosome amplification; however, after a 120h exposure, 0.1, 0.15 or 0.2 $\mu\text{g}/\text{cm}^2$ zinc chromate induced 13.9, 18 and 21.3 percent of interphase cells with greater than 2 centrosomes (Figure 4B). The majority of aberrant interphase cells had three or four centrosomes (Figure 4C). There was a statistically significant positive relationship for interphase cell centrosome amplification with time at all concentrations and with concentration at 48, 72, 96 and 120 h ($p < 0.05$).

Normal mitotic cells have two centrosomes that orient in a bipolar fashion and generate bipolar mitotic spindles, which ensure proper alignment of chromosomes along the metaphase plate (Figure 5A(1–4)). In contrast, Figure 5A(5–8) depicts an aberrant mitotic cell after zinc chromate exposure with multiple centrosomes, multipolar spindle formation and disorganized DNA. Figure 5A (9–12) also depicts an aberrant mitotic cell with multiple centrosomes but the mitotic spindles exhibit a more bipolar orientation and the DNA is more well-organized along the metaphase plate. Background centrosome amplification in mitotic cells ranged between 1–3 percent. Consistent with the interphase cells and the aneuploidy data, no centrosome amplification in mitotic cells was observed after 24 h, but there was an increase in centrosome amplification with time and concentration. Exposure to 0.1, 0.15 and 0.2 $\mu\text{g}/\text{cm}^2$ zinc chromate for 120 h induced 21, 33 and 46 percent mitotic cells with greater than 2 centrosomes. No mitotic cells with one centrosome were observed. There was a statistically significant increase in mitotic cell centrosome amplification with time at all zinc chromate

concentrations and with concentration at 48, 72, 96 and 120 h ($p < 0.05$). In contrast to interphase cells, the majority of the aberrant mitotic cells had 3, 4, 5 and 6 centrosomes and the range of centrosome number was greater with mitotic cells having as many as fourteen centrosomes (Figure 5C).

Four possible mechanisms for centrosome amplification exist, all involving alterations in the number of centrioles (24). These include: multiple centrosome duplication cycles in one cell cycle, failure of cytokinesis, centriole splitting and acentriolar centrosome formation (23). Accordingly, we investigated the number of centrioles in the centrosomes using an antibody against centrin. We investigated centriole number in both interphase and mitotic cells. In interphase cells, each centrosome normally has one or two centrioles depending on the phase of the cell cycle (Figure 6A(1–4)). We found that after zinc chromate exposure, there was a time-dependent increase in cells with abnormal centrosomes (greater than 2 centrosomes) and normal centrioles (all centrosomes with one centriole or two centrioles) in interphase cells (Figure 6A(5–8), Figure 6B).

A normal mitotic cell has two centrosomes and each centrosome will have two centrioles (Figure 6C(1–4)). After zinc chromate exposure, we observed a time-dependent increase in mitotic cells with abnormal centrosomes and normal centrioles and abnormal centrosomes and abnormal centrioles (Figure 6C(5–8), Figure 6D). Cells with abnormal centrin were defined as having at least one centrosome with no centrioles, one centriole, or more than two centrioles. A statistically significant increase in cells with amplified centrosomes and normal centrin and amplified centrosomes and abnormal centrin was observed with time after exposure to 0.2 $\mu\text{g}/\text{cm}^2$ zinc chromate ($p < 0.05$).

Chronic exposure to zinc chromate induces a G2 arrest

DNA damage-induced prolonged S phase and G2 phase arrests have been linked to centrosome amplification. During a prolonged S or G2 arrest, the DNA cycle and the centrosome cycle can become uncoupled resulting in multiple rounds of centrosome amplification during the DNA cycle arrest (38–39). Therefore, we wanted to determine if chronic exposure to zinc chromate arrested the cell cycle. We found that after chronic exposure to zinc chromate, G1 levels remained relatively constant while S phase levels declined and G2/M levels increased (Figure 7A–C). A 120 h exposure to 0.2 $\mu\text{g}/\text{cm}^2$ zinc chromate induced 59 percent G1 cells, 14 percent S phase cells and 28 percent G2/M cells while the control had 62 percent G1 cells, 24 percent S phase cells and 15 percent G2/M cells (Figure 7A–C). In order to pinpoint whether the arrest was occurring at G2 or M, we performed a mitotic index after chronic zinc chromate exposure. We found that the mitotic index significantly decreased after zinc chromate (Figure 7D), confirming that the arrest was occurring at G2. There was a statistically significant decrease in S phase with zinc chromate concentration at all time points ($p < 0.05$) and a statistically significant increase in G2/M arrest with time at all zinc chromate concentrations and with concentration at 48, 72, 96 and 120 h ($p < 0.05$). All zinc chromate concentrations and time points exhibited a statistically significant decrease in mitotic index with time and concentration ($p < 0.05$).

Discussion

Particulate hexavalent chromium compounds are known human lung carcinogens (1–4); however, the underlying carcinogenic mechanisms remain unknown. The best mechanistic model appears to be one that involves genomic instability (*reviewed in* 7), however little is known about the ability of the particulate or soluble Cr(VI) compounds to induce genomic instability. This mechanism includes both MIN and CIN, consistent with observations that both effects are simultaneously observed generally in lung cancer cells (8). For the particulate Cr(VI) compounds, the only genomic instability data focus on CIN. Particulate compounds have

been shown to induce structural CIN manifested as chromosomal aberrations in chromate workers, and in cultured human lung cells (9,13–19,41–42). In particular, these studies have largely focused on lead chromate (13–14,16,18,41–42) and studies of numerical CIN have only considered lead chromate (20–21). Thus, it is uncertain if this mechanism applies to chromate compounds generally or if it is specific to lead chromate.

Zinc chromate is a particularly potent particulate Cr(VI) carcinogen and an industrially important Cr(VI) compound, but only a few studies have considered it, reporting elevated carcinogenic risk in human workers, tumors in experimental animals and genotoxicity and oxidative stress in human lung cells (19,26–34). We extend these observations and find that zinc chromate induces numerical CIN in both a time- and concentration-dependent manner, consistent with the phenotype generally seen in lung cancers (22).

Numerical CIN is potentially caused by a number of mechanisms. The spindle assembly checkpoint functions between metaphase and anaphase and serves to prevent inappropriate separation of the sister chromatids (23). A ‘go’ signal (i.e. one that indicates it is time to progress to anaphase) occurs when all of the kinetochores are attached to microtubules and the microtubule fibers achieve sufficient levels of tension (23). Alterations of the expression of spindle assembly checkpoint genes have been observed generally in lung cancer (22), though they have not yet been studied in tumors from chromate workers. Disruption of the spindle assembly checkpoint is understudied for metals. Data from lead chromate show that it can induce numerical CIN by disrupting the spindle assembly checkpoint (21). We found that chronic exposure to zinc chromate induced concentration- and time-dependent increases in spindle assembly checkpoint bypass, in the form of centromere spreading, premature centromere division and premature anaphase. When we compare lead chromate and zinc chromate based on administered concentration, zinc chromate induces more spindle assembly checkpoint bypass than lead chromate. Exposure to 0.1 $\mu\text{g}/\text{cm}^2$ zinc chromate for 120 h induced 12% cells with evidence of spindle assembly checkpoint bypass while 0.1 $\mu\text{g}/\text{cm}^2$ lead chromate for 120 h induced 3% cells with evidence of spindle assembly checkpoint bypass (21). The only other reports of metal effects on the spindle assembly checkpoint found that arsenic induced spindle assembly checkpoint bypass and permanent CIN (43–44). Our data are consistent with these previous reports for lead chromate and arsenic and suggest that spindle assembly checkpoint bypass may be a common mechanism for particulate chromate compounds in general and may be an important general mechanism for metal carcinogenesis.

Centrosomes function to separate the chromosomes into two daughter cells (25). Centrosome amplification can result in multipolar spindle formation and division leading to numerical CIN (25). Centrosome amplification has been observed generally in lung cancers (22,45), though it has not been considered in tumors from chromate workers. Disruption of centrosome dynamics is also understudied for metals. Data from lead chromate show that it can induce numerical CIN by increasing the number of centrosomes (20). Our data show that zinc chromate induced both a concentration- and time-dependent increase in cells with increased centrosome number in both interphase and mitotic cells. When we compare zinc chromate and lead chromate based on administered concentration, we find that zinc chromate induces more centrosome amplification than lead chromate. Exposure to 0.1 $\mu\text{g}/\text{cm}^2$ zinc chromate for 120 h induced centrosome amplification in 14% of interphase cells and 21% of mitotic cells while the same concentration of lead chromate induced centrosome amplification in 7% of interphase cells and 3% of mitotic cells (20). Other previous reports show that arsenic and methylmercury can induce centrosome amplification (20,46–50) and numerical CIN (20,48). Our data are consistent with these previous reports for lead chromate, arsenic and methylmercury and suggest that centrosome amplification may be a common mechanism for particulate chromate compounds in general and may be an important general mechanism for metal carcinogenesis.

Centrosome amplification can occur through multiple mechanisms including multiple centrosome duplication cycles in one cell cycle, failure of cytokinesis, centriole splitting and acentriolar centrosome formation (24). The mechanism of centrosome amplification can be determined by staining the centrioles in the centrosome. If multiple centrosome duplication cycles occurs during a cell cycle, all of the centrosomes should all have two centrioles. If failure of cytokinesis occurs, these centrosomes would have two centrioles, but the cell would be binucleated. If the cell has undergone centriole splitting at least one centrosome would have only one centriole. Finally, if acentriolar centrosome formation occurs, at least one centrosome would have no centrioles.

Currently, no studies have investigated the effects of metals on centrioles. We found that zinc chromate increased the number of cells with amplified centrosomes and normal centrioles in both interphase and mitotic cells and also the number of cells with amplified centrosomes and abnormal centrioles in mitotic cells. These data suggest that more than one mechanism is involved in chromate-induced centrosome amplification. Since there was an increase in cells with amplified centrosomes and normal centrioles but no increase in binucleated cells (data not shown), some cells underwent multiple centrosome duplication cycles in one cell cycle to generate amplified centrosomes. Studies show that cells can undergo multiple centrosomes duplication cycles during a DNA damage-induced G2 arrest (38). We observed a persistent G2 arrest after chronic zinc chromate exposure suggesting that the cells with amplified centrosomes and normal centrin may have undergone multiple duplication cycles during a zinc chromate-induced G2 arrest. The cells in the amplified centrosomes and abnormal centrioles category exhibited centrosomes with no centrioles, centrosomes with one centriole and/or centrosomes with more than two centrioles. This suggests that cells with centrosomes with no centrioles underwent acentriolar centrosome formation and cells with centrosomes with one centriole underwent uncontrolled centriole splitting. This is the first study to show that chromium can induce multiple centrosome duplication cycles in one cell cycle, uncontrolled centriole splitting and acentriolar centrosome formation as mechanisms of centrosome amplification.

It is uncertain how particulate chromate compounds induce centrosome amplification and spindle assembly checkpoint bypass. One hypothesis, albeit untested, is that continual exposure to chromium induces chronic DNA damage resulting in a prolonged G2 arrest (46–47). DNA damage-induced G2 arrest can lead to centrosome amplification (36). Consistent with this mechanism, chromate compounds only induce DNA double strand breaks in G2 phase and do not induce breaks in G1 or S phase (11,18,34). Other chromate compounds have been reported to induce G2 arrest after shorter (i.e. less than 24 h) exposures (12,34,51–52). We extend those results here to include longer exposures (i.e. 48–120 h), indicating that zinc chromate can induce a prolonged G2 arrest, consistent with this mechanism. The percentage of cells in S phase and the mitotic index both decrease after zinc chromate exposure indicating that it does not induce an S phase or mitotic arrest. There is no apparent effect on the percentage of G1 phase cells suggesting that zinc chromate may not induce a G1 phase arrest. However, it is interesting to note that even though zinc chromate does not induce an accumulation of cells in G1 phase, the percentage of cells in G1 phase remains comparable to control and does not decrease. This observation could suggest there may also be a zinc chromate effect on the transition between G1 to S because with an increase in G2 phase and a decrease in mitosis, we would expect to see a concurrent decrease in G1. These results are consistent with our previous report showing that a 24 h exposure to zinc chromate induces a G2 arrest, with a decrease in the percentage of cells in S phase but no effect on G1 cells at the low and middle dose range although higher doses decreased both S and G1 (34).

Increased aneuploidy, centrosome amplification and spindle assembly checkpoint bypass occurred only after longer exposures to zinc chromate; these phenotypes were not observed

after only a 24 h exposure. These data suggest that more chronic exposures to zinc chromate are required for these phenotypes to appear. However, the timing of the initial cellular disruption remains unknown. It is possible that the reason why we observe a steady increase in aneuploidy, centrosome amplification and spindle assembly checkpoint bypass with time is because a subset of mitotically active cells are slowly re-emerging from an initial cell cycle arrest that occurred early in treatment. Future work is aimed at addressing this question to determine how many cell cycle divisions these abnormal mitotic cells have undergone.

The physico-chemical mechanism for particulate chromate-induced toxicity has only been studied with lead chromate. Those studies show that lead chromate dissolves outside the cells into its respective lead cation and chromate anion (40,53–54). The lead cation and chromate anion then enter the cell and the internalized chromium induces cytotoxicity, chromosome aberrations and DNA double strand breaks (13–18), while the lead ion does not appear to play a role in lead chromate-induced toxicity (13,35,55). In addition, the internalized lead chromate particles appear to remain in vacuoles and do not contribute to lead chromate-induced cytotoxicity or genotoxicity (13,35,54–56). Thus, the toxicity of particulate lead chromate appears to be from soluble Cr. These results also appear to extend to numerical CIN as data show that lead ions do not induce centrosome amplification and that particle-cell contact is not required for spindle assembly checkpoint bypass suggesting that it is the soluble Cr ion that is responsible for centrosome amplification and spindle assembly checkpoint bypass (20–21). It is interesting to note, as discussed above, that zinc chromate was more potent than the values reported for lead chromate. This observation is consistent with the epidemiology and whole animal studies that zinc chromate is a more potent carcinogen. This difference could indicate that zinc is playing a role in inducing spindle assembly checkpoint bypass or centrosome amplification; however, no studies have investigated the effects of increased zinc levels on centrosome amplification or spindle assembly checkpoint bypass. Alternatively, it could be that more chromate anion gets into cells after zinc chromate exposure making it more potent.

We hypothesize that a mechanism for particulate chromate-induced carcinogenesis would start with particles impacting in the lung and dissolving outside the cell into their respective ions, which then enter the cell. Once inside the cell, Cr(VI) is reduced to Cr(III) producing reactive intermediates and free radicals. Cr(III), one of the reactive intermediates, and/or reactive oxygen species can damage the DNA inducing chromosome aberrations, DNA adducts and strand breaks. Chronic exposure induces continual DNA damage and a prolonged G2 arrest resulting in centrosome amplification and spindle assembly checkpoint bypass and ultimately aneuploidy. Further work is aimed at determining the mechanistic links between the G2 arrest and the centrosome amplification and spindle assembly checkpoint bypass and determining the molecular events that induce such a wide range of centriole aberrations.

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Figure 1. Chronic Exposure to Zinc Chromate Induces Cytotoxicity in Human Lung Cells

This figure shows that chronic exposure to zinc chromate induces concentration- and time-dependent decreases in relative survival. All doses and time points are statistically different from control ($p < 0.05$). There was a statistically significant negative relationship with time at all zinc chromate concentrations and with concentration at all time points ($p < 0.05$).

Figure 2. Chronic Exposure to Zinc Chromate Induces Aneuploidy in Human Lung Cells

This figure shows that chronic exposure to zinc chromate increases aneuploidy in a concentration- and time-dependent manner. A) Percentage of aneuploid metaphases (greater or less than 46 chromosomes). There was a statistically significant positive relationship with time at all zinc chromate concentrations and with concentration at 72, 96 and 120 h ($p < 0.05$). B) Percentage of hypodiploid metaphases (less than 46 chromosomes). There was a statistically significant positive relationship with concentration at 96 and 120 h and with time at 0.2 $\mu\text{g}/\text{cm}^2$ zinc chromate ($p < 0.05$). C) Percentage of hyperdiploid metaphases (greater than 46 but less than 92 chromosomes). There was a statistically significant positive relationship with concentration at 120 h and with time at 0.15 $\mu\text{g}/\text{cm}^2$ zinc chromate ($p < 0.05$). D) Percentage of tetraploid metaphase (92 chromosomes). There was a statistically significant positive relationship with time at all zinc chromate concentrations and with concentration at 48 h ($p < 0.05$). * Statistically different from control ($p < 0.05$).

Figure 3. Chronic Exposure to Zinc Chromate Causes an Increase in Cells with Evidence of Spindle Assembly Checkpoint Bypass

This figure shows that chronic exposure to zinc chromate increases spindle assembly checkpoint bypass in a time- and concentration-dependent manner. A) These pictures are representative examples of centromere spreading (1), premature centromere division (2) and premature anaphase (3). The arrows indicate chromosomes with centromere spreading and the circles represent chromosomes that completely separated from their sister chromatid. B) This graph shows that chronic exposure to zinc chromate induces concentration- and time-dependent increases in centromere spreading, premature centromere division and premature anaphase. There was a statistically significant increase in total spindle assembly checkpoint bypass with time at all zinc chromate concentrations and with concentration at 96 and 120 h ($p < 0.05$). ^a Cells in premature anaphase are statistically different from control ($p < 0.05$), ^b cells in premature centromere division are statistically different from control ($p < 0.05$), ^c cells with centromere spreading are statistically different from control ($p < 0.05$).

Zinc Chromate Concentration (ug/cm ²)	Time (h)	Normal Centrosome Number		Percent of Interphase Cells with Aberrant Centrosome Number					
		1	2	3	4	5	6	7	8
Control 0.2	24	13	84	0.8	1.4	0.2	0.07	0	0
		13	84	1.2	1.2	0.1	0.07	0	0
Control 0.2	48	13	84	1.3	1.7	0.2	0	0	0
		11	81	4	3.6	0.3	0.1	0.1	0
Control 0.2	72	13	84	0.8	1.7	0.2	0.1	0	0
		8	81	4.3	6.4	0.5	0.1	0	0.03
Control 0.2	96	13	83	1.3	2.5	0.4	0.17	0.03	0
		9	76	6.1	8.0	0.6	0.3	0.1	0.03
Control 0.2	120	13	82	1.5	2.8	0.5	0.13	0	0
		8	70	8.5	10.8	1.4	0.53	0.07	0

Figure 4. Chronic Exposure to Zinc Chromate Induces Centrosome Amplification in Interphase Cells

This figure shows that chronic exposure to zinc chromate induces a concentration- and time-dependent increase in centrosome amplification in interphase cells. A) Pictures 1–4 represent a normal interphase cell with two centrosomes. Pictures 5–8 represent an aberrant interphase cell with four centrosomes. Blue (DAPI) represents the DNA, red (Alexa 555) represents the centrosomes (gamma-tubulin) and green (FITC) represents the microtubules (alpha-tubulin). B) This graph shows that the percentage of interphase cells with greater than 2 centrosomes increases in a concentration- and time-dependent manner after zinc chromate exposure. There was a statistically significant positive relationship with time at all concentrations and with concentration at 48, 72, 96 and 120 h ($p < 0.05$). *Statistically different from control ($p < 0.05$). C) This table shows the spectrum of centrosome number in interphase cells chronically treated with 0.2 ug/cm² zinc chromate.

C)

Zinc Chromate Concentration (ug/cm ²)	Time (h)	Normal Centrosome Number	Percent of Mitotic Cells with Aberrant Centrosome Number											
			3	4	5	6	7	8	9	10	11	12	13	14
Control	24	99	0.7	0.7	0	0	0	0	0	0	0	0	0	0
		99	0.7	0	0.3	0	0	0	0	0	0	0	0	0
Control	48	98	1	1	0	0	0	0	0	0	0	0	0	0
		90	4	3	2	0	0.7	0.3	0	0	0	0	0	0
Control	72	98	1	0.7	0	0	0	0	0	0	0	0	0	0
		82	7	7	2.3	1.3	0	0	0	0	0	0	0	0
Control	96	99	0.3	1	0	0	0	0	0	0	0	0	0	0
		71	9	11	5.3	2.7	1	0.3	0	0	0	0	0	0
Control	120	97	2.7	0.7	0	0	0	0	0	0	0	0	0	0
		54	10	14	8	6	4	2.7	0	0.7	0	0	0.3	0.7

Figure 5. Chronic Exposure to Zinc Chromate Induces Centrosome Amplification in Mitotic Cells

This figure shows that chronic exposure to zinc chromate induces a concentration- and time-dependent increase in centrosome amplification in mitotic cells. A) These pictures represent cells with normal or aberrant mitotic centrosomes. Pictures 1–4 represent a normal mitotic cell with two centrosomes and bipolar spindle formation. Pictures 5–8 represent an aberrant mitotic cell with 4 centrosomes, multipolar spindle formation and disorganized DNA. Pictures 9–12 represent an aberrant mitotic cell with six centrosomes but bipolar spindle formation. Blue (DAPI) represents the DNA, red (Alexa 555) represents the centrosomes (gamma-tubulin) and green (FITC) represents the microtubules (alpha-tubulin). B) This graph shows that the percentage of mitotic cells with greater than 2 centrosomes increases in a concentration- and time-dependent manner after zinc chromate exposure. There was a statistically significant increase in centrosome amplification with time at all zinc chromate concentrations and with concentration at 48, 72, 96 and 120 h ($p < 0.05$). *Statistically different from control ($p < 0.05$). C) This table shows the spectrum of centrosome number in mitotic cells chronically treated with 0.2 ug/cm² zinc chromate.

Figure 6. Chronic Exposure to Zinc Chromate Induces Centrosome Amplification with both Normal and Abnormal Centrioles

This figure shows that chronic exposure to zinc chromate induces amplified centrosomes with both normal and abnormal centrioles. A) These pictures represent normal and aberrant interphase cells. Pictures 1–4 represent a normal interphase cell with two centrosomes and one centriole per centrosome. Pictures 5–8 represent an aberrant interphase cell with four centrosomes and one centriole per centrosome. Blue (DAPI) represents the DNA, red (DyLight 549) represents the centrosomes (gamma-tubulin) and green (DyLight 488) represents the centrioles (centrin). B) This graph shows that interphase cells with amplified centrosomes and normal centrin increases in a time-dependent manner ($p < 0.05$). * Cells with amplified centrosomes and normal centrin are statistically different from control ($p < 0.05$). C) These pictures represent normal and aberrant mitotic cells. Pictures 1–4 represent a normal mitotic cell with two centrosomes and two centrioles in each centrosome. Pictures 5–8 represent an

aberrant mitotic cell with amplified centrosomes and normal centrin. This cell has four centrosomes and two centrioles in each centrosome. D) This graph shows that mitotic cells with amplified centrosomes and abnormal centrin and amplified centrosomes and normal centrin increase after exposure to 0.2 ug/cm² zinc chromate. A statistically significant increase in cells with amplified centrosomes and normal centrin and amplified centrosomes and abnormal centrin was observed with time after exposure to 0.2 ug/cm² zinc chromate (p<0.05). ^a Cells with amplified centrosomes and abnormal centrin are statistically different from control (p<0.05), ^b cells with normal centrosomes and abnormal centrin are statistically different from control (p<0.05), ^c cells with amplified centrosomes and normal centrin are statistically different from control (p<0.05).

Figure 7. Chronic Exposure to Zinc Chromate Induces a G2 Arrest

This figure shows that chronic exposure to zinc chromate induces a G2 arrest. A) Percent of cells in G1. Treated cells were not statistically different from control. B) Percent of cells in S phase. All concentrations and time points were statistically different from control ($p < 0.05$). There was a statistically significant decrease in S phase with zinc chromate concentration at all time points ($p < 0.05$). C) Percent of cells in G2/M. There was a statistically significant increase in G2/M arrest with time at all zinc chromate concentrations and with concentration at 48, 72, 96 and 120 h ($p < 0.05$). Exposure to 0.15 ug/cm² zinc chromate for 48 and 120 h and 0.2 ug/cm² zinc chromate for 48, 72, 96 and 120 h were statistically different from control. D) Mitotic index. All zinc chromate concentrations and time points exhibited a statistically

significant decrease in mitotic index with time and concentration ($p<0.05$). All concentrations and time points were statistically different from control ($p<0.05$).