Chronic Exposure to Particulate Chromate Induces Premature Centrosome Separation and Centriole Disengagement in Human Lung Cells

Julieta Martino,*,† Amie L. Holmes,*,† Hong Xie,*,† Sandra S. Wise,*,†,‡ and John Pierce Wise Sr *,†,‡,1

*Wise Laboratory of Environmental and Genetic Toxicology and †Department of Applied Medical Sciences, Maine Center for Toxicology and Environmental Health, University of Southern Maine, Portland, Maine 04104; and ‡Department of Pharmacology and Toxicology, University of Louisville, Louisville, Kentucky 40068

1To whom correspondence should be addressed at Department of Pharmacology and Toxicology, University of Louisville, 505 S. Hancock St, CTRB 522, Louisville, KY 40068. Fax: 502-852-7868. E-mail: john.wise@louisville.edu.

ABSTRACT

Particulate hexavalent chromium (Cr(VI)) is a well-established human lung carcinogen. Lung tumors are characterized by structural and numerical chromosome instability. Centrosome amplification is a phenotype commonly found in solid tumors, including lung tumors, which strongly correlates with chromosome instability. Human lung cells exposed to Cr(VI) exhibit centrosome amplification but the underlying phenotypes and mechanisms remain unknown. In this study, we further characterize the phenotypes of Cr(VI)-induced centrosome abnormalities. We show that Cr(VI)-induced centrosome amplification correlates with numerical chromosome instability. We also show chronic exposure to particulate Cr(VI) induces centrosomes with supernumerary centrioles and acentriolar centrosomes in human lung cells. Moreover, chronic exposure to particulate Cr(VI) affects the timing of important centriolar events. Specifically, chronic exposure to particulate Cr(VI) causes premature centriole disengagement in S and G2 phase cells. It also induces premature centrosome separation in interphase. Altogether, our data suggest that chronic exposure to particulate Cr(VI) targets the protein linkers that hold centrioles together. These centriolar linkers are important for key events of the centrosome cycle and their premature disruption might underlie Cr(VI)-induced centrosome amplification.

Key words: chromium; CIN; centrosome amplification; centriole disengagement; centrosome separation

Lung cancer is the second most common type of cancer and the leading cause of cancer death in the United States (ACS, 2014). Although cigarette smoking accounts for most cases, about 9%–15% of cases can be attributed to environmental and occupational exposures such as asbestos, tar, soot, radiation, and metals (Alberg and Samet, 2003). Hexavalent chromium [Cr(VI)] is a metal widely used in industry because of its hardness, anticorrosive properties and bright colored salts. Its main uses are in stainless steel production, chrome plating, anticorrosive and refractory applications, as a pigment in paints and dyes, and in leather tanning (Sarnhart, 1997). Cr(VI) is also a common environmental pollutant and ranked among the top 20 hazardous substances by the Environmental Protection Agency and the Agency for Toxic Substances and Disease Registry (ATSDR, 2014). Moreover, Cr(VI) is also a well-established human lung carcinogen (IARC, 1990).

Lung tumors are generally characterized by complex karyotypes with abnormal number of chromosomes (Masuda and Takahashi, 2002). This numerical chromosome instability (CIN) can be caused by defects in sister chromatid cohesion, kinetochore structure or function, aberrant cell cycle checkpoints and abnormal centrosome function (Lengauer et al., 1998). Centrosomes are organelles that nucleate and organize microtubules to form the mitotic spindle that segregates sister
chromatids. Structurally, a centrosome is composed of a pair of centrioles surrounded by proteins that form the pericentriolar material. Upon division, normal cells inherit 1 centrosome which is duplicated only once before mitosis. However, tumors and tumor-derived cell lines commonly exhibit centrosome amplification (ie, cells with >2 centrosomes) (Chan, 2011; Fihan et al., 1998). Centrosome amplification has been correlated extensively with numerical CIN (Pukasawa, 2005) because supernumerary centrosomes can generate abnormal division of sister chromatids through multipolar spindles and merotelic microtubule–kinetochore attachments (Ganem et al., 2009).

Previous studies have shown that Cr(VI) induces centrosome amplification and numerical CIN (Holmes et al., 2006a, 2010b; Wise and Wise, 2012; Xie et al., 2007). Other toxic metals such as arsenic, organic mercury and titanium dioxide can also induce centrosome amplification (Holmes et al., 2010a). However, although these studies established that it occurs, a deeper understanding of the impacts of metals on centrosomes has not been considered. In this study, we delved deeper into Cr(VI)-induced centrosome amplification. Our data show that Cr(VI) induces premature centriole disengagement and premature centrosome separation, which correlate with centrosome amplification and numerical CIN previously observed for Cr(VI) (Holmes et al., 2006a, 2010b; Wise and Wise, 2012; Xie et al., 2007). Our data provide novel targets for Cr(VI) toxicity and offers mechanistic insights into Cr(VI)-induced centrosome amplification.

MATERIALS AND METHODS

Chemicals and reagents. Zinc chromate (CAS # 13530-65-9) was purchased from Alfa Aesar (A18178, Ward Hill, Massachusetts). Dulbecco’s Minimal Essential Medium and Ham’s F-12 (DMEM/ F-12) 50:50 mixture, Dulbecco’s Phosphate Buffered Saline 1 x (PBS), penicillin/streptomycin and glutarGRO were purchased from Mediatech, Inc (Manassas, Virginia). Cosmic calf serum was purchased from HyClone (Logan, Utah). Trypsin/EDTA (0.25%) and Gurr’s buffer were purchased from Gibco (Grand Island, New York). Sodium pyruvate was purchased from Lonza (Walkersville, Maryland). Tissue culture dishes, flasks, and plasticware were purchased from BD (Franklin Lakes, New Jersey). Demecolcine, potassium chloride, magnesium sulfate, EGTA, PIPES were purchased from Sigma-Aldrich (St. Louis, Missouri). Methanol, acetone and acetic acid were purchased from J.T. Baker (Phillipsburg, New Jersey). Giemsa stain was purchased from Alfa Aesar (A18178, Ward Hill, Massachusetts). Prolong Gold Antifade Reagent with DAPI and Alexa Fluor Cy3 and Cy5 conjugates, Alexa Fluor 488 and 568 secondary antibodies were purchased from Invitrogen (Carlsbad, California). DyLight secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, Pennsylvania).

Cells and cell culture. WTHBF-6, an hTERT immortalized clonal cell line derived from primary human bronchial fibroblasts (Wise et al., 2004), was used in all experiments. This cell line has normal growth parameters, a normal stable karyotype and a cytotoxic and clastogenic response to metals similar to primary cells (Wise et al., 2004). WTHBF-6 cells were maintained as an adherent subconfluent monolayer in DMEM/F-12 supplemented with 15% cosmic calf serum, 0.2 mM L-alanyl-L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.1 mM sodium pyruvate. All experiments were maintained in a 37°C humidified incubator with 5% CO2.

Preparation of zinc chromate and cell treatments. Zinc chromate was administrated as a suspension of particles in cold sterile water as previously described (Xie et al., 2009). Logarithmically growing cells were seeded and allowed to rest for 48 h. The media was changed and cells were treated for 24, 72, and 120 h with concentrations of 0.1, 0.15, and 0.2 μg/cm2 of zinc chromate. These concentrations correspond to 0.12, 0.18, and 0.24 ppm, respectively, and as such are within the ranges of environmental exposures and well below the levels found in lungs of workers occupationally exposed to Cr(VI) (ie, 2.6–104 ppm) (ATSDR, 2012). These concentrations have also been shown to induce DNA double strand breaks and chromosomal aberrations and are cytotoxic to WTHBF-6 cells (Holmes et al., 2010b; Qin et al., 2014).

Numerical CIN analysis. Numerical CIN was determined by counting the number of chromosomes in solid stained metaphases. Chromosome preparation was performed as previously described (Wise et al., 2002). A minimum of 100 metaphases were analyzed per concentration/time point and at least 3 experiments were performed.

Centrosome amplification analysis. Cells seeded on chamber slides were treated with zinc chromate as described above. After treatment, cells were rinsed with microtubule stabilizing buffer (3 mM EGTA, 50 mM PIPES, 1 mM magnesium sulfate, 25 mM potassium chloride), fixed with −20°C methanol for 10 min and permeabilized with 0.05% Triton X-100 for 3 min. After blocking, cells were incubated with an anti-γ-tubulin antibody (T6557, Sigma-Aldrich), washed with PBS and incubated with Alexa Fluor 555 secondary antibody. This was followed by washing and incubation with anti-x-tubulin–FITC conjugated antibody (F2168, Sigma-Aldrich). Cells were washed and coverslips were mounted with DAPI. Centrosome numbers in 100 mitotic and 1000 interphase cells were analyzed per concentration/time point using fluorescence microscopy. At least 3 independent experiments were performed.

Centrosome separation measurement. Cells were seeded, treated and harvested as described for centrosome amplification analysis. Pictures of 100 interphase cells per concentration/time point were taken using a Nikon Eclipse Ti confocal microscope. The distance between centrosomes that were within the same plane was measured using the Nikon NIS Elements software. Centrosomes were classified as separated when the distance between them was >2 μm (Meraldi and Nigg, 2001). Each experiment was repeated independently at least 3 times.

Centrin analysis. Cells seeded on glass chamber slides were treated with zinc chromate. After treatment, cells were fixed as described for centrosome amplification analysis. After blocking, cells were incubated with anti-centrin (gift from Dr Jeffrey Salisbury, Mayo Clinic, Rochester, Minnesota) and anti-γ-tubulin antibodies, washed with 0.05% Triton X-100 and incubated with DyLight 549 and 488 secondary antibodies. Cells were washed with 0.05% Triton X-100 and coverslips were mounted with DAPI. Centrosome and centriole numbers were analyzed in 200 interphase and 50 mitotic cells per concentration/time point using fluorescence microscopy. At least 3 independent experiments were performed.
Centriole number data were also used to quantify the number of cells with evidence of centriole disengagement. Interphase cells in G1 with engaged centrioles have 1 centrosome with 2 centrioles, while cells with evidence of disengagement have 2 centrosomes, each with a single centriole. Cells in S, G2, and mitosis with engaged centrioles have 2 centrosomes with 2 centrioles each, while cells with evidence of disengagement either have 3 centrosomes, 1 with 2 centrioles and 2 with single centrioles; or 4 centrosomes each with a single centriole.

For the earlier centrin analysis, the assumption was made that all interphase cells with 2 centrioles were in G1 phase. To confirm the findings of this assay, we also counted centriole numbers in cells stained with cyclin D1. Cyclin D1 is expressed at high levels in G1 cells, where it localizes to the nucleus (Ballen et al., 1993). Cells were seeded, harvested and stained as above with the addition of a 1 h incubation with anti-Cyclin D1 antibody (sc-8396, Santa Cruz Biotechnology, Inc, Dallas, Texas) at room temperature. Centriole numbers were analyzed in 100 interphase cells with nuclear cyclin D1 staining per concentration/time point. Three independent experiments were performed.

Centriole disengagement analysis. Cells seeded on glass chamber slides were treated with zinc chromate. After treatment, cells were washed with PBS and fixed with –20 °C methanol and acetone for 10 and 1 min, respectively. Cells were rehydrated in PBS and incubated with anti-centrin (#94-1624, Millipore, Billerica, Massachusetts), anti-C-Nap1 (14498-1-AP, Proteintech, Chicago, Illinois) and anti-γ-tubulin antibodies. Cells were then washed, incubated with isotype-specific DyLight 488, Alexa Fluor 594 and Alexa Fluor 633 secondary antibodies, and coverslips were mounted with DAPI. A total of 100 interphase and 50 mitotic cells with a normal centrin number (2 or 4 centrin foci) were analyzed per concentration/time point. The ratio of centrin to C-Nap1 foci was used to confirm centriole disengagement (Tsou and Stearns, 2006). Cells with a 1:1 ratio were classified as having disengaged centrioles, while cells with a 2:1 ratio were classified as having their centrioles engaged. Three independent experiments were performed.

Statistics. Values were expressed as the mean ± SEM (standard error of the mean) of triplicate experiments. Two-way ANOVA analyses (α = 0.05) were used to determine the statistical significance of the variables in consideration (ie, zinc chromate concentration and exposure time). ANOVA analyses were followed by Tukey tests to determine the differences in pairs of means among the different groups. All statistical analyses were performed using GraphPad Prism Version 6.05 (GraphPad Software, Inc, La Jolla, California).

RESULTS

Chronic Exposure to Zinc Chromate Induces Numerical CIN that Correlates with Centrosome Amplification

No increase in numerical CIN was observed after 24 h exposure to zinc chromate. However, exposure to 0.1, 0.15, and 0.2 μg/cm² zinc chromate for 72 or 120 h induced 14%, 21%, and 24% metaphases with numerical CIN, respectively, and 28%, 40%, and 44% metaphases with numerical CIN, respectively (p < .05) (Fig. 1A). This increase in numerical CIN correlated with a time- and concentration-dependent increase in centrosome amplification in mitotic cells (p < .05) (Figs. 1A and 1B). No increase in the percent of cells with > 2 centrosomes was observed after 24 h exposure, while exposure to 0.1, 0.15, and 0.2 μg/cm² zinc chromate for 72 or 120 h induced 9%, 15%, and 18%, and 21%, 33%, and 46% of mitotic cells with centrosome amplification, respectively (Figs. 1A and 1B).

Chronic Exposure to Zinc Chromate Induces Cells with Supernumerary Centriole Numbers

Centrosomes are comprised of a pair of centrioles surrounded by a matrix of proteins called the pericentriolar material. Upon cytokinesis, normal cells inherit 1 centrosome with 2 centrioles, which is duplicated once during S phase. Hence, perturbations in the number of centrioles can impact centrosome number. We evaluated the impact of zinc chromate by using a centriole marker, centrin, to quantify centriole numbers in interphase and mitotic cells.

Normal interphase cells exhibit 3 patterns of centrosome numbers. Normal G1 cells have either 1 centrosome with 2 centrioles, or 2 centrosomes with 1 centriole each. In contrast, normal S/G2 cells have 2 centrosomes with 2 centrioles each (Fig. 2A). Using these normal patterns as a guide, zinc chromate only reduced the number of S/G2 cells and did not affect the G1 cells (Fig. 2B). For example, after 24 h exposure to 0.2 μg/cm² zinc
chromate, 44% of cells had 2 centrosomes with 2 centrioles each, which decreased to 35% and 36% of cells after 72 and 120 h exposures, respectively (Fig. 2B). This decrease was not statistically significant but was accompanied by an increase in the number of cells with supernumerary centrioles (Fig. 2B). At 24 h exposure to 0.2 μg/cm² zinc chromate, there were 1% of cells with supernumerary centrioles, while after 120 h there were 8% (Fig. 2B). Approximately 1%–2% of interphase cells had an abnormal number of 3 centrioles but this percentage did not change after chromate treatment.

Next, we analyzed centriole number in mitotic cells. Normal mitotic cells have 2 centrosomes with 2 centrioles each (Fig. 2C). Consistent with the interphase cell data, there was a statistically significant decrease in the number of mitotic cells with a normal number of centrioles (Fig. 2D). No decrease was observed after a 24 h exposure to 0.2 μg/cm² zinc chromate. However, after 120 h, the percent of cells with normal centrioles decreased to 67%. There was also a statistically significant increase in the percent of mitotic cells with supernumerary centrioles after chronic exposure (Fig. 2D). No increase was observed after 24 h exposure to 0.2 μg/cm² zinc chromate. After 120 h, the percent of cells increased to 30%. At 24 h, approximately 1%–2% of mitotic cells had 3 centrioles, and this percentage did not change after 72 and 120 h treatments. All the effects on centriole numbers in mitotic cells were consistent with the trends seen in interphase cells. Overall, our data show that chronic exposure to zinc chromate causes supernumerary centrioles in mitotic cells.

Chronic Exposure to Zinc Chromate Induces Premature Centriole Disengagement

While quantifying the number of centrioles in interphase and mitotic cells, we noticed many cells had a normal number of centrioles that exhibited extra centrosomes. This outcome is possible if the centrioles from a centrosome become disengaged from each other. In normal cells, centrioles disengage at the end of mitosis or early G1 (Agircan et al., 2014). Premature centriole disengagement (ie, occurring in S or G2 phase or in mitosis before anaphase) results in centrosome amplification because disengaged centrioles can undergo reduplication thus increasing centriole and centrosome numbers (Tsou and Stearns, 2006). Our observations that zinc chromate causes supernumerary centrioles, suggested abnormal centriole disengagement might be of concern.

We quantified centrosome and centriole numbers to determine the number of cells with centriole disengagement. As shown in Fig. 3A, cells with engaged centrioles show the following phenotypes: G1 cells will have 1 centrosome with 2 centrioles. S, G2, or mitotic cells will have 2 centrosomes with 2 centrioles each. Disengaged centrioles can form a centrosome, thus, in contrast to cells with engaged centrioles, cells with disengaged centrioles will show these phenotypes: G1 cells will have 2 centrosomes each with 1 centriole (Fig. 3A). For S, G2, or mitotic cells, if centrioles from only 1 centrosome disengage, these cells will have 3 centrosomes, 2 with 1 centriole each, and the unaffected centrosome with 2 centrioles (Fig. 3A). However, if centrioles from both centrosomes disengage,
these cells will have 4 centrosomes, each with 1 centriole (Fig. 3A).

Particulate Cr(VI) increased centriole disengagement in S and G2 cells (Fig. 3B). After exposure to 0.2 \( \mu g/cm^2 \) zinc chromate for 120 h, there were 14% of S/G2 cells with evidence of centriole disengagement, respectively (\( p < .05 \)), compared with 3% after a 24 h exposure. Particulate Cr(VI) also increased centriole disengagement in mitotic cells (Fig. 3C). Specifically, after 120 h exposure to 0.2 \( \mu g/cm^2 \) zinc chromate, 12% of cells exhibited centriole disengagement (\( p < .05 \)), compared with 0% after a 24 h exposure. This increase in centriole disengagement was paralleled by a decrease in cells with engaged centrioles (\( p < .05 \)) (Fig. 3C). By contrast, zinc chromate did not increase centriole disengagement in G1 cells (Fig. 3B).

During normal cell division normal centriole disengagement can occur in late mitosis or early G1. It is interesting to note that approximately 50% of untreated G1 cells showed evidence of disengagement suggesting that WTHBF-6 cells exhibit disengagement in G1. Moreover, if disengagement occurred in late mitosis, we would expect to see most G1 cells with evidence of centriole disengagement which was not the case.

For the centriole disengagement analysis in interphase cells, we classified cells with 2 centrioles to be in G1 phase, and cells with 4 centrioles to be in S or G2 phase. This classification is based on normal centrosome dynamics. To confirm the outcomes of this approach, we also directly measured G1 cells using cyclin D1, a G1 phase cell cycle marker (Baldin et al. 1993).

We quantified centriole numbers in cyclin D1-positive cells and found that indeed, cells with 2 centrioles are in G1 phase (Fig. 3D). Moreover, exposure to 0.2 \( \mu g/cm^2 \) zinc chromate for 24, 72, and 120 h did not change the percent of cyclin D1-positive cells with 2 centrioles confirming the centriole disengagement results. Interestingly, we observed some mitotic cells with engaged centrioles had acentriolar centrosomes. Acentriolar centrosomes lack centrioles and can arise from overexpression of pericentriolar material components or fragmentation of the pericentriolar material (Fukasawa, 2005; Maiato and Logarinho, 2014). No acentriolar centrosomes were observed after 24 h exposure to 0.2 \( \mu g/cm^2 \) zinc chromate. In contrast, the percent of cells with acentriolar centrosomes increased to 11% after a 120 h exposure (\( p < .05 \)) (Fig. 3C).

**Centrin:C-Nap1 Ratios Confirm Cr(VI)-Induced Premature Centriole Disengagement in Interphase Cells**

We confirmed the centrin data indicating Cr(VI) exposure causes premature centriole disengagement, by analyzing the ratio of centrin to C-Nap1 foci (Tsou and Stearns, 2006). C-Nap1 (ie, CEP250, centrosomal Nek2-associated protein 1) is a protein found at the free proximal end of centrioles. When 2 centrioles are engaged, they have 2 centrin foci (1 from each centriole) and 1 C-Nap1 focus at the free proximal end of the mother centriole (Tsou and Stearns, 2006). Thus, engaged centrioles have a 2:1 centrin:C-Nap1 foci ratio. By contrast, disengaged centrioles have 2 free proximal ends, 1 from the mother centriole and 1
from the now disengaged daughter centriole creating a 2:2 centrin.C-Nap1 foci ratio (Fig. 4A).

Zinc chromate induced a time- and concentration-dependent increase in centriole disengagement in interphase cells with 4 centrioles (S or G2 cells). No effect was seen after 24 h exposure. However, a 120 h exposure to 0.1, 0.15, and 0.2 μg/cm² zinc chromate induced centriole disengagement in 25%, 40%, and 51% of cells, respectively (p < .05) (Fig. 4B). These outcomes are consistent with the analysis of centrosome and centriole numbers from previous sections. Interestingly, cells with only 2 centrioles (G1 cells) also had increased centriole disengagement after chronic exposure to zinc chromate, although this increase was not statistically significant (Fig. 4C). In mitotic cells, there was a slight increase in cells with disengaged centrioles but it was not statistically significant (Fig. 4D). In addition, centriole disengagement in interphase cells correlated with centrosome amplification (Fig. 4E).

**Chronic Exposure to Zinc Chromate Induces Premature Centrosome Separation**

Duplicated centrosomes formed in S phase are normally held together by a protein linker. This linker is severed at the G2/M transition and allows centrosomes to separate in prophase so they can be positioned in a bipolar fashion (Slangy et al., 1995). Depletion of linker proteins by siRNA caused increased centriole disengagement when cells were exposed to a DNA damaging agent (Conroy et al., 2012). This outcome suggests this linker may be involved in protecting centriole engagement. Because Cr(VI) is a strong DNA damaging agent (Holmes et al., 2006b; Wise et al., 2002; Xie et al., 2005, 2009) and causes centriole disengagement, we hypothesized that Cr(VI) exposure might also disrupt this linker. Hence, we measured centrosome separation in interphase cells under the premise that if this linker is severed, centrosomes would premature separate in interphase (Bahe et al., 2005; Faragher and Fry, 2003; Mardin and Schiebel, 2012; Mayor et al., 2000). Using gamma-tubulin as a marker for centrosomes, we measured the distance between pairs of centrosomes. Centrosomes were classified as separated if the distance between them was > 2 μm (Meraldi and Nigg, 2001) (Fig. 5A).

After a 24 h exposure, there was no effect on the percent of cells with centrosome separation. However, exposure to zinc chromate for 120 h caused a statistically significant increase in the percent of interphase cells with centrosome separation (Fig. 5B). Specifically, a 24 h exposure to 0.1, 0.15, and 0.2 μg/cm² zinc chromate induced centrosome separation in 15%, 15%, and 13% of cells, respectively. By contrast, after 120 h exposure, the percent of cells with centrosome separation at these concentrations increased to 30%, 33%, and 36%, respectively (p < .05) (Fig. 5B). Moreover, increased centrosome separation correlated with increased centrosome amplification and premature centriole disengagement observed after chronic exposure to zinc chromate (Figs. 5C and 5D). Overall, our data show chronic exposure to zinc chromate induces premature centrosome separation, suggesting the linker that holds centrosomes together may be severed prematurely or not formed properly.

**DISCUSSION**

Centrosome amplification is a common feature of tumors (Chan, 2011). Supernumerary centrosomes cause missegregation of chromosomes and numerical CIN (Ganem et al., 2009). CIN is a driving force for tumorigenesis and its understanding is key to the development of anticancer therapies (Jallepalli and Lengauer, 2001). Chemical carcinogens, such as Cr(VI), can cause centrosome amplification (Holmes et al., 2010b), but the impact of metals on centrosomes remains poorly studied.

This is the first article to show Cr(VI) causes premature centriole disengagement in S/G2 cells (Fig. 4B). The marked increase in centriole disengagement observed after Cr(VI) treatment strongly suggests Cr(VI)-induced centrosome amplification could be mainly mediated through premature centriole disengagement. Normal centriole disengagement occurs during late mitosis or early G1 (Agircan et al., 2014). Centriole disengagement is important because it serves as a licensing factor for centriole duplication (Tsou and Stearns, 2006). However, if disengagement is premature (i.e., during S, G2 or in mitosis before anaphase) cells can undergo multiple rounds of centriole duplication that lead to centrosome amplification (Douthwright and Sluder, 2014; Fukasawa, 2005; Inanç et al., 2010; Saladin et al., 2009).

The ability of metals to induce centriole disengagement has not been previously reported, however, our results are in agreement with studies that have shown premature centriole disengagement in human cells after exposure to DNA damaging agents such as radiation and doxorubicin (Conroy et al., 2012; Douthwright and Sluder, 2014; Saladin et al., 2009). Altogether, these data suggest premature centriole disengagement might be a general response to DNA damaging agents.

We also observed Cr(VI) treatment induced premature centrosome separation in interphase cells (Fig. 5B). Centrosome separation normally occurs in mitosis during prophase and serves to position centrosomes for bipolar spindle formation (Tanenbaum and Medema, 2010). Delayed centrosome separation causes an increase in mitotic time and chromosome segregation errors (Kaseda et al., 2012; Mchedlishvili et al., 2012; Silkworth et al., 2012), while premature centrosome separation could favor the survival of genetically unstable cells due to a more efficient formation of the mitotic spindle (Mardin et al., 2013). Given that Cr(VI) causes CIN (Holmes et al., 2008; Wise et al., 2008) premature centrosome separation could be involved in the survival of damaged cells.

Furthermore, Conroy et al. (2012) showed that DNA damage, centrosome separation, and centriole disengagement are inter-related. Ionizing radiation-induced centriole disengagement further increased if cells were depleted of rootletin and C-Nap1, proteins that tether centrosomes together and thus prevent centrosome separation (Bahe et al., 2005; Mayor et al., 2000; Yang et al., 2006). This study suggests that the inter-centrosome linker may also protect mother and daughter centrioles from disengagement. Because Cr(VI) causes premature centriole disengagement and centrosome separation, an appealing hypothesis is that Cr(VI) targets the protein linkers that hold centrosoles and centrosomes together. This may be mediated through direct interaction with the linker proteins or by deregulation of the signaling pathways that control the formation and disruption of these linkers.

Exposure to Cr(VI) also induced an increase in acentriolar centrosomes. This was only observed in mitotic cells (Fig. 3C). Acentriolar centrosomes can arise from pericentriolar material fragmentation caused by spindle and chromosomal forces (Maia and Logarinho, 2014). Moreover, spindle forces are also involved in centrosome separation (Raaijmakers et al., 2012; Slangy et al., 1995; Tanenbaum et al., 2009). It is tempting to speculate that centrosome separation induced by Cr(VI) could partially be caused by increasing the activity of the motor proteins that generate these microtubules forces.
FIG. 4. Chronic exposure to Cr(VI) induces premature centriole disengagement. This figure shows that chronic exposure to zinc chromate induces premature centriole disengagement in S/G2 cells. A, Representative schematics and pictures of cells with centrosomes with engaged and disengaged centrioles. B, Centriole disengagement in S/G2 cells. 
*Cells with disengaged centrioles are statistically different from the control (p < .05). 
*Cells with engaged centrioles are statistically different from the control (p < .05). C, Centriole disengagement in G1 cells. D, Centriole disengagement in mitotic cells. 
*Cells with centriole disengagement and centrosome amplification caused by zinc chromate show a similar increase and trend. 
*Cells with centrosome amplification are statistically different from the control (p < .05).
Overall, using centrin as a marker for centrioles, we have described the multiple centriolar defects caused by chronic exposure to the carcinogen Cr(VI). These data further define the centrosome amplification phenotype in cells exposed to Cr(VI) and show that multiple mechanisms might be at play in carcinogen-induced centrosome amplification. Future research will focus on the molecular components of centrosome amplification.

**FUNDING**

This work was supported by the National Institute of Environmental Health Sciences (ES016893 to J.P.W.) and the Maine Center for Toxicology and Environmental Health. The content is solely the responsibility of the presenters and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health.

**ACKNOWLEDGMENTS**

The authors thank Geron Corporation for the use of the hTERT materials and Shouping Huang and Christy Gianios, Jr for administrative and technology support. They also thank Dr. Jeffrey Salisbury from the Mayo Clinic (Rochester, Minnesota) for providing us with centrin antibody and Dr. W. Douglas Thompson for advice on statistical analyses.

**REFERENCES**


