Homologous Recombination Repair Signaling in Chemical Carcinogenesis: Prolonged Particulate Hexavalent Chromium Exposure Suppresses the Rad51 Response in Human Lung Cells

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ABSTRACT

The aim of this study was to focus on hexavalent chromium, [Cr(VI)], a chemical carcinogen and major public health concern, and consider its ability to impact DNA double strand break repair. We further focused on particulate Cr(VI), because it is the more potent carcinogenic form of Cr(VI). DNA double strand break repair serves to protect cells against the detrimental effects of DNA double strand breaks. For particulate Cr(VI), data show DNA double strand break repair must be overcome for neoplastic transformation to occur. Acute Cr(VI) exposures reveal a robust DNA double strand break repair response, however, longer exposures have not been considered. Using the comet assay, we found longer exposures to particulate zinc chromate induced concentration-dependent increases in DNA double strand breaks indicating breaks were occurring throughout the exposure time. Acute (24 h) exposure induced DNA double strand break repair signaling by inducing Mre11 foci formation, ATM phosphorylation and phosphorylated ATM foci formation, Rad51 protein levels and Rad51 foci formation. However, longer exposures reduced the Rad51 response. These data indicate a major chemical carcinogen can simultaneously induce DNA double strand breaks and alter their repair and describe a new and important aspect of the carcinogenic mechanism for Cr(VI).

Key words: hexavalent chromium; chromate; particulate Cr(VI); Rad51; DNA double strand break repair; homologous recombination repair

DNA repair protects cells against the DNA damaging effects of chemical carcinogens and must be overcome for a complete carcinogen to cause cancer. For example, DNA double strand breaks are one of the most dangerous DNA lesions that may lead to massive loss of genetic information and cell death. They must be efficiently repaired to maintain genome integrity and functionality. Particulate Cr(VI) is a known human lung carcinogen that can induce DNA double strand breaks after acute (<24 h) exposures (Holmes et al., 2008; Wise and Wise, 2012; Wise et al., 2008; Xie et al., 2005). Moreover, studies show a DNA double strand break repair deficient phenotype is required for particulate Cr(VI)-induced neoplastic transformation in human lung cells (Xie et al., 2008). Because normal cells have a robust DNA double strand break repair system, a carcinogen, like Cr(VI),
would have to alter this repair in some fashion to induce neoplastic transformation. However, there are no data indicating such an event occurs and, in general, the consideration has been that either a dose high enough to cause sufficient damage to overwhelm the repair system would be necessary, a mutation in a repair gene might occur or coexposure to another carcinogen might be necessary.

In general, DNA double strand breaks are recognized and subsequently repaired by two primary pathways: nonhomologous end-joining (NHEJ) and homologous recombination (HR) (Iliakis et al., 2004). HR ensures an error-free repair by using the undamaged sister chromatid or homologous chromosome as a template. A set of proteins, H2A.X, MRN (Mre11/Rad50/Nbs1) complex, ATM, 53BP1, Rad51, Rad54, BRCA1/2, and more, play important roles in HR (Ciccia and Elledge, 2010). It is generally accepted that double strand breaks are recognized by MRN binding to the damaged DNA end. This binding then recruits and activates ATM which phosphorylates several intracellular partners to activate the downstream signal-transduction cascades (Uziel et al., 2003).

Strand-exchange protein, Rad51, is a key mediator of HR (Sung and Robberson, 1995). Rad51 protein forms the helical nucleoprotein filament to promote DNA strand exchange and stimulate DNA pairing activity, the basic steps of homologous recombination (Sung and Robberson, 1995).

Studies in Chinese hamster cells show loss of HR-mediated DNA double strand break repair results in increased Cr(VI)-induced chromosome instability (Stackpole et al., 2007), whereas loss of NHEJ-mediated repair did not have this effect (Camyre et al., 2007). Studies in human lung cells show exposures to particulate Cr(VI) for over 24 h induces chromosome instability (Holmes et al., 2006a, 2010; Wise et al., 2006) suggesting HR repair might be affected after longer Cr(VI) exposures.

HR-related proteins such as Mre11, ATM, NBS1, and Rad51 are elevated in cells treated with particulate Cr(VI) after acute exposure (i.e., <24 h) (Bryant et al., 2006; Xie et al., 2005, 2008). However, there are no data describing how this repair pathway responds after prolonged exposure to particulate Cr(VI) as, so far, all of the cell culture studies that investigate DNA double strand break formation and response have focused on shorter exposure times (i.e., <24 h) (Holmes et al., 2008; Wise and Wise, 2012; Wise et al., 2008). Therefore, this study considers longer exposure times and investigates the formation of DNA double strand breaks and the responses of repair proteins in the HR pathway to determine if particulate Cr(VI) can alter break repair while inducing breaks, thus, overcoming this protective DNA repair pathway.

MATERIALS AND METHODS

Chemicals and reagents. Zinc chromate was purchased from Alfa Aesar (CAS no. 13530-65-9; ACS reagent minimum 98% purity, Alfa Aesar, Ward Hill, MA). Dulbecco’s Modified Eagle’s Medium and Ham’s F-12 (DMEM/F-12) 50:50 mixture, glutarGRO and 1X phosphate-buffered saline (PBS) were purchased from Medi-atech Inc. (Herndon, VA). Bovine serum albumin (BSA) and cosmic calf serum (CCS) were purchased from Hyclone (Logan, UT). Gurr’s buffer, trypsin/EDTA, sodium pyruvate and penicillin/streptomycin were purchased from Life Technologies (Grand Island, NY). Demecolcine and potassium chloride (KCl) were purchased from Sigma-Aldrich (St Louis, MO). Giema stain was purchased from Biomedical Specialties Inc. (Santa Monica, CA). Acetic acid, methanol, tris-base, EDTA, glycine, and sodium acetate were purchased from J.T. Baker (Phillipsburg, NJ). Tissue culture dishes, flasks, and plasticware were purchased from BD Inc. (Acton, MA). Antibodies for immunofluorescence staining included: anti-phospho-ATM was purchased from Abcam (Cambridge, UK); anti-Mre11 was purchased from Calbiochem (Darmstadt, Germany), anti-Rad51 was purchased from Santa Cruz (Dallas, TX), and Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 555 goat anti-mouse IgG were purchased from Life Technologies. Antibodies for Western blot: anti-Mre11 and B-actin were purchased from Novus (Littleton, CO), antiphosphorylated ATM and anti-Rad51 were purchased from Abcam, and anti-ATM was purchased from Cell Signaling (Danvers, MA).

Cells and cell culture. WTHBF-6 cells, an h-TERT immortalized clonal cell line with growth parameters and a Cr(VI) response similar to the primary human bronchial fibroblasts (PHBF) they were derived from, were used in all experiments as previously described (Wise et al., 2004). WTHBF-6 cells were maintained as monolayer in DMEM/F-12 supplemented with 15% CCS, 1% L-glutAro, 0.1mM sodium pyruvate, and 1% penicillin/streptomycin. All experiments were maintained in a 37°C, humidified incubator with 5% CO₂.

Preparation of particulate Cr(VI). Epidemiologic, animal, and cell culture studies show the water insoluble particulate Cr(VI) compounds are more potent than the water soluble ones (Holmes et al., 2008; Wise and Wise, 2012; Wise et al., 2008). Zinc chromate appears to be the most potent of the particulate Cr(VI) compounds, based on epidemiologic studies of lung cancer cases in chromium-exposed workers (Levy and Venitt, 1986). Thus, we used zinc chromate as a representative particulate Cr(VI) compound. It was administered as a suspension of particles in cold sterile water as previously described (Xie et al., 2009). Cells were treated with a concentration range of 0.1–0.3 μg/cm². The cytotoxicity of these doses are described in Holmes et al. (2010) and show that as expected longer exposures to genotoxic concentrations induces more toxicity than shorter exposure times, but many cells survive.

Neutral comet assay. DNA double strand breaks were measured using a single cell gel electrophoresis assay as previous described with slight adjustments (Xie et al., 2005). Briefly, logarithmically growing cells were seeded into six-well plates and treated with zinc chromate accordingly. At the end of treatment, cells were washed, collected, and resuspended in PBS. The cell suspension was then mixed with agarose at 1:10 ratio and evenly spread onto Trevigen Comet slides. After complete solidification on ice, slides were immersed in a freshly prepared lysis solution (2.5M NaCl, 100mM EDTA, 10mM Tris, pH 10) containing additional 1% Triton X-100 at 4°C for 30 min, followed by 1 mg/ml proteinase-K incubation for 2 h at 37°C. Electrophoresis was carried out at 21 V (1 V/cm) for 10 min in freshly prepared electrophoresis buffer (300mM sodium acetate, 100mM Tris, pH 9.0). Finally, slides were immersed in DNA precipitation buffer (0.15M NH₄Ac, ethanol) for 20 min followed by fixation in 70% ethanol and stained with SYBR green. All the steps described above were conducted under reduced light to prevent spurious DNA damage. Comet images were captured using an Olympus fluorescence microscope equipped with a SensiCam. Image analysis was carried out by Comet Assay IV software (Perceptive Inc., UK). Tail moment (tail length × percentage of migrated DNA) was adopted as a measurement to quantify DNA damage. One hundred randomly selected cells were analyzed for each treatment concentration. Each experiment was repeated three times.
Immunofluorescence staining and microscopy. DNA double strand break repair foci were detected using immunofluorescence as previously described (Xie et al., 2009). Briefly, logarithmically growing cells were seeded into each well of an eight-well chamber slide and treated with zinc chromate accordingly. At harvest, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 5% goat serum. Cells were then incubated with anti-Mre11, anti-Rad51, or anti-phosphorylated ATM at 4°C overnight and then incubated with Alexa Fluor 488 goat anti-rabbit IgG at room temperature for 1 h. Nuclei were counterstained with Prolong Gold antifade reagent with 4',6-Diamidino-2-Phenylindole (DAPI). Foci were viewed and counted with an Olympus fluorescent microscope. One hundred nuclei per each treatment concentration were analyzed. Results were expressed as the percentage number of cells with >5 foci or >10 foci based on background levels such that negative controls had 5% or less of cells with this level. Each experiment was repeated at least three times.

Clastogenicity assay. Clastogenicity was determined by measuring the amount of chromosomal damage in treatment groups compared with controls as previously described (Wise et al., 2002). Briefly, logarithmically growing monolayer cells were seeded into 100 mm dishes and treated with zinc chromate accordingly. One hour prior to the harvest time, 0.1 ug/ml demecolcine was added to arrest cells in metaphase. Cells were then collected, resuspended in hypotonic solution of 0.075M KCl, and fixed with 3:1 methanol:acetic acid. Finally, the cells were dropped on a clean wet glass slide and uniformly stained using a 5% Giemsa stain in Gurr’s buffer. One hundred metaphase per concentration were analyzed in each experiment. Results were expressed as a percentage of metaphases with chromosome damage (the metaphase is used as a comparison unit) and as the total chromosome aberrations observed in 100 metaphases (the chromosome is used as a comparison unit). Each experiment was repeated at least three times.

Western blot. Nuclear extracts were prepared according to our published methods (Xie et al., 2005, 2008) with some minor modifications. Briefly, logarithmically growing cells were seeded into 100 mm dishes and treated with zinc chromate. At the end of treatment, cells were washed, collected, and resuspended in buffer A (10mM HEPES, 10mM KCl, and 0.1mM EDTA) supplemented with protease inhibitors, phosphatase inhibitors, and 0.8% IGEPAL. After incubation on ice for 15 min, nuclei were collected and lysed with buffer B (20mM HEPES, 0.4M NaCl, 1mM EDTA, and 10% glycerol) containing protease inhibitors and phosphatase inhibitors while vigorously shaking on ice for 2 h. Protein concentrations were then determined and equal samples were resolved by either 3–8% tris-acetate or 10% Bis-Tris SDS-PAGE gels. Immunoblots were probed with the following primary antibodies: anti-rabbit phosphorylated ATM, anti-rabbit ATM, anti-rabbit Rad51 and anti-Mre11. Equal protein loading was confirmed by β-actin. Immunoblot signals were detected using ECL plus reagent and images were obtained with Storm Image System (Amersham Biosciences, Piscataway, NJ).

Statistics. Values were expressed as the mean ± SEM (standard error of the mean) of triplicate experiments. The Student’s t-test was used when comparing two variables, and one-way ANOVA was used when comparing different time points. The Student’s t-test was used to calculate p-values to determine the statistical significance of difference in means for each pair of concentrations. A 95% confidence interval for the difference in means of each pair of concentrations was constructed based on the Student’s t-distribution.

RESULTS

Chronic Exposure to Particulate Cr(VI) Induces DNA Double Strand Breaks in Human Lung Cells

For DNA double strand break repair to matter for chemical carcinogens, the chemical must induce DNA double strand breaks. Because particulate Cr(VI) induced increasing amounts of chromosome instability after prolonged exposures (Holmes et al., 2006a,b; Wise et al., 2006), we evaluated the impact of these exposures on DNA double strand breaks in human lung cells. We found zinc chromate induced a concentration-dependent increase in comet tail formation measured as tail moment (Fig. 1). For example, a 24-h exposure to 0, 0.1, 0.2, and 0.3 ug/cm² zinc chromate produced a tail moment of 1.4, 2.4, 2.7, and 3.2, respectively. Exposures for 48–72 h induced a concentration-dependent increase in the breaks, but at similar level as 24 h exposure indicating there was no time-dependent increase in the breaks. At 96 h, there was an increase in the amount of breaks that returned, after 120 h exposure, to the levels seen at 24–72 h, although this increase was not statistically significant. However, the 96 h time point is statistically higher than control (p < 0.025).

It is generally accepted that unrepaired DNA double strand breaks can lead to structural chromosome abnormalities such as chromosomal breakage, deletions, translocations, and gross rearrangements. Thus, we measured the ability of zinc chromate to induce structural chromosome abnormalities over time. This endpoint confirmed the comet assay results. We found longer exposures to zinc chromate induced a concentration-dependent increase in chromosome damage (Figs. 2A and 2B). The spectrum of chromosome damage included chromatid gaps and breaks, isochromatid gaps and breaks, dicentric chromosomes, double minutes, acentric fragments, and chromatid exchanges, consistent with the production of DNA double strand breaks (data not shown). The lowest exposure, consistent with the 96 h spike in comet assay data, showed a time-dependent increase after 96 h exposure (Fig. 2C). In contrast to the results in the comet assay,
FIG. 2. Prolonged exposure to particulate Cr(VI) induces chromosome damage in human lung cells. This figure shows zinc chromate induces persistent chromosome damage. Data represent an average of three experiments. Error bars = standard error of the mean. *Total chromosome aberrations = the total number of chromosome aberrations in 100 metaphases. (A) Percentage of metaphase cells with chromosome damage. All of the zinc chromate concentrations at each treatment time point were significantly ($p < 0.025$) different from control. 0.1, 0.15, and 0.2 ug/cm$^2$ zinc chromate at 120 h, 0.15 and 0.2 ug/cm$^2$ zinc chromate at 96 h were significantly ($p < 0.025$) different from 24 h treatment. 0.2 ug/cm$^2$ zinc chromate at 120 h was significantly ($p < 0.01$) different from all other time points. (B) Total chromosome aberrations in 100 metaphases. All of the zinc chromate concentrations at each treatment time point were significantly ($p < 0.01$) different from control. 0.1, 0.15, and 0.2 ug/cm$^2$ zinc chromate at 120 h, 0.15 and 0.2 ug/cm$^2$ zinc chromate at 96 h, and 0.2 ug/cm$^2$ zinc chromate at 72 h were significantly ($p < 0.025$) different from 24 and 48 h treatments. 0.2 ug/cm$^2$ zinc chromate at 120 h was significantly ($p < 0.01$) different from all other time points. (C) After exposure to 0.1 ug/cm$^2$ zinc chromate, the time course of chromosome damage mirrors the DNA double strand break time course measured by comet tail moment, except at 120 h when there is a drop in the amount of breaks. Data are extracted from Figures 1A and 2B with negative control values subtracted.

Chronic Exposure to Particulate Cr(VI) Suppresses Homologous Recombination Repair Signaling in Human Lung Cells

Having determined prolonged exposures to particulate Cr(VI)-induced DNA double strand breaks, we next sought to understand the impact of these exposures on DNA repair to see if this carcinogen could alter this repair. Double strand breaks are primarily repaired by the NHEJ and HR repair pathways (Iliakis et al., 2004; Lee and Paull, 2005). Our previous studies show that, of these two pathways, HR is more important after Cr(VI) exposure (Camyre et al., 2007; Stackpole et al., 2007; Xie et al., 2008) so we focused on this pathway.

HR signaling and repair involve a variety of proteins cooperatively participating in different steps of a signaling cascade that generally can be grouped into three steps: (1) sensing the damage (sensor), (2) transducing and amplifying the repair signal (transducer), and (3) carrying out the repair (effector). We selected a representative protein for each step. Mre11 acts as an early sensor and activates ATM, the master signal transducer (So et al., 2005; Uziel et al., 2003). Rad51 is responsible for initiating the single strand invasion and homology pairing in sister chromatids during the effector stage of HR (Sung and Robberson, 1995). Thus, to evaluate the impact of particulate Cr(VI) on the HR repair pathway, we chose Mre11, ATM, and Rad51 as representatives of the sensor, transducer, and effector steps, respectively, based on their central and critical roles in the different steps of HR signaling (Fig. 3, Supplementary Fig. 1).

Zinc chromate did not significantly alter Mre11 protein levels, but it did increase Mre11 foci formation in a concentration-dependent manner after a 24, 72, or 120 h exposure. For example, 24 h exposure to 0.0, 0.1, 0.2, and 0.3 ug/cm$^2$ zinc chromate induced an average of 4.3, 8, 10.3, and 15% of cells with Mre11 foci, respectively. In addition, there was a time-dependent increase in Mre11 foci formation. For example, exposure to 0.3 ug/cm$^2$ zinc chromate induced an average of 15, 24.6, and 40% of cells with Mre11 foci after 24, 72, and 120 h exposure, respectively.

Zinc chromate also increased pATM foci in a time- and concentration-dependent manner. For example, exposure to 0.3 ug/cm$^2$ zinc chromate...
FIG. 3. Prolonged exposure to particulate Cr(VI) alters Rad51 response in human lung cells. This figure shows that chronic exposure to zinc chromate causes alteration of double strand break protein levels and foci formation measured by Western blot and immunofluorescence assays, respectively. Data represent an average of three experiments for foci formation and at least two experiments for Western blot. Error bars = standard error of the mean. *pATM and Mre11 percentage of cells with >5 foci, Rad51 percentage of cells with >10 foci. (A) Mre11 nuclear protein levels measured by Western blotting. (B) The ratio of phosphorylated-ATM to nonphosphorylated-ATM protein levels, in the nucleus measured by Western blotting. (C) Rad51 nuclear protein levels measured by Western blotting. (D) Percentage of cells with >5 Mre11 nuclear foci measured by immunofluorescence. 0.1, 0.2, and 0.3 ug/cm² zinc chromate at 120 h; 0.2 and 0.3 ug/cm² zinc chromate at 72 h, and 0.3 ug/cm² zinc chromate at 24 h were significantly (p < 0.05) different from controls. 0.2 and 0.3 ug/cm² zinc chromate at 120 h and 0.3 ug/cm² zinc chromate at 24 h were significantly (p < 0.05) different from 0.1 ug/cm² zinc chromate. 0.1, 0.2, and 0.3 ug/cm² zinc chromate at 120 h were significantly (p < 0.05) different from 24 and 72 h treatments. (E) Percentage of cells with >5 phosphorylated-ATM nuclear foci measured by immunofluorescence. 0.1, 0.2, and 0.3 ug/cm² zinc chromate at 24 and 120 h and 0.2 and 0.3 ug/cm² zinc chromate at 72 h were significantly (p < 0.05) different from controls. 0.2 ug/cm² zinc chromate at 24, 72, and 120 h were significantly (p < 0.05) different than 0.1 ug/cm² zinc chromate. 0.1, 0.2, and 0.3 ug/cm² zinc chromate at 120 h were significantly (p < 0.05) different from 24 and 72 h treatments. (F) Percentage of cells with >10 Rad51 nuclear foci measured by immunofluorescence. At 24, 48, and 72 h, all concentrations were significantly different from control (p < 0.05). At 96 h, 0.1 and 0.2 ug/cm² zinc chromate were significantly different from control (p < 0.05). (G) Combination of three patterns of Mre11 foci (sensor), phosphorylated ATM foci (transducer), and Rad51 foci (effector) after exposure to 0.1 ug/cm² zinc chromate. (H) Combination of three patterns of Mre11 foci (sensor), phosphorylated ATM foci (transducer), and Rad51 foci (effector) after exposure to 0.2 ug/cm² zinc chromate. (I) Combination of three patterns of Mre11 foci (sensor), phosphorylated ATM foci (transducer), and Rad51 foci (effector) after exposure to 0.3 ug/cm² zinc chromate. (J) Representative images of Mre11 Foci. All images are control or after 0.2 ug/cm² zinc chromate exposure at the time points of 24, 72, and 120 h. (K) Representative images of pATM foci colocalized with yH2A.X foci, a common marker of a double strand break. All images are control or after 0.2 ug/cm² zinc chromate exposure at the time points of 24, 72, and 120 h. (L) Representative images of Rad51 foci after zinc chromate exposure. All images are control or after 0.2 ug/cm² zinc chromate exposure at the time points of 24, 72, and 120 h.
Prolonged exposure to particulate Cr(VI) increases cytoplasmic Rad51 localization in human lung cells. This figure shows that chronic exposure to zinc chromate induces Rad51 cytoplasmic retention. (A) Representative images of three different states of Rad51 protein localization in human lung cells. (I) Rad51 exhibits a diffused state in both the cytoplasm and nucleus under the nonstress condition. (II) Rad51 relocates to form nuclear foci in response to DNA double strand breaks. (III) Rad51 aggregates in cytoplasm during continuous exposure to zinc chromate. FITC (top) stains for Rad51, DAPI (second row) stains for DNA, DIC (third row) shows the whole cell and the merged image (bottom) shows that the Rad51 aggregates are in the cytoplasm. (B) Quantification of cells with Rad51 cytoplasmic retention. 0.1 ug/cm² zinc chromate at 72 and 120 h, 0.2 and 0.3 ug/cm² zinc chromate at 96 and 120 h were significantly (p < 0.05) different from control. 0.3 ug/cm² zinc chromate at 120 h was significantly (p < 0.05) different from 0.1 and 0.2 ug/cm² zinc chromate. 0.1 ug/cm² zinc chromate at 72 and 120 h was significantly (p < 0.05) different from 24 and 48 h treatments, whereas 0.2 and 0.3 ug/cm² zinc chromate at 96 and 120 h were significantly (p < 0.005) different from all other treatment time points. Data represent an average of three experiments. Error bars = standard error of the mean.

**DISCUSSION**

DNA double strand breaks are a particularly concerning type of DNA damage as the ability to repair them is compromised by the loss of both strands resulting in the lack of a template to copy and restore the original DNA. To address this problem cells engage HR repair which finds a homologous chromosome or DNA sequence to copy and restore the template. Thus, because of its high fidelity, HR becomes a significant hurdle that chemical carcinogens must overcome to induce carcinogenesis.

Hexavalent chromium is a well-known human lung carcinogen (Wise et al., 2002). Solubility plays a key role in Cr(VI) carcinogenicity, with the particulate compounds being the most potent (Holmes et al., 2008; Wise and Wise, 2012; Wise et al., 2002, 2008). It is hypothesized that the carcinogenic properties of particulate Cr(VI) result from the chronic release of Cr(VI) ions at the target tissue leading to the production of persistent and mispaired DNA damage (Holmes et al., 2008; Wise and Wise, 2012; Wise et al., 2008).

Previously, we showed acute (24 h) exposure to particulate Cr(VI)-induced DNA double strand breaks and chromosome...
damage in a concentration-dependent manner, but did not induce chromosome instability (Holmes et al., 2006a,b; Wise et al., 2006, 2010; Xie et al., 2008, 2009). However, longer exposures did induce chromosome instability (Holmes et al., 2006a; Wise et al., 2006), and since HR repair protects cells against Cr(VI)-induced chromosome instability (Bryant et al., 2006; Stackpole et al., 2007; Xie et al., 2005, 2008), we investigated further and found these exposures also inhibited the Rad51 response.

In particular, longer particulate Cr(VI) exposures alter the effector arm of the DNA double strand break repair signaling and impact the protein levels and localization of Rad51 to repair foci. Rad51 is a central player in HR repair, mediating the pairing and strand exchange between homologous DNA sequences during repair (Sung and Robberson, 1995). Our data confirmed acute exposure to particulate Cr(VI) activates HR signaling by inducing ATM phosphorylation, Rad51 protein levels, and foci formation. But, after 48–96 h of zinc chromate exposure, Rad51 foci fail to increase in response to treatment as they did during the initial 24 h of treatment. After 120 h of exposure, the Rad51 foci response actually diminishes below baseline levels.

The cause of this Rad51 effect is uncertain, but is probably because at 48 h of zinc chromate exposure, Rad51 begins to aggregate in the cytoplasm instead of the nucleus. This aggregation increased in both a time- and concentration-dependent manner. The underlying cause for the aggregation is also unknown, but is likely a failure to transport Rad51 protein into the nucleus or the export of Rad51 out of the nucleus. Rad51 does not have a nuclear localization signal and appears to be dependent on Rad51C or BRCA2 for its transport into the nucleus (Gilde-meister et al., 2009; Jeyasekharan et al. 2013). Rad51 does have a nuclear export signal that is usually masked by its interactions with BRCA2 (Jeyasekharan et al. 2013). Thus, one possibility, albeit untested, is that Cr interferes with Rad51C or Brca2 interactions with Rad51.

It is notable that this loss of Rad51 response occurs, despite the ongoing presence of DNA double strand breaks. Our data show longer particulate Cr(VI) exposures induce a concentration-dependent increase in breaks at each exposure time, but the amount of breaks (measured by the comet assay) do not appreciably increase over time except for a peak at 96 h in the comet assay. The amount of DNA double strand breaks in treated cells and the number of cells with DNA double strand breaks after longer exposures remained constant between time points.

The explanation for the lack of time-dependent response is uncertain. Previously, we showed prolonged exposures to particulate Cr(VI) induce continually increasing amounts of Cr ions in these cells (Holmes et al., 2006a; Wise et al., 2006), thus, one would expect to see damage increase with time as Cr(VI) continues to enter the cell. One explanation might be that the persistence of the DNA double strand break levels observed in the current study reflects an equilibrium between break formation and repair, resulting in a near constant level of damage. In this scenario, particulate Cr(VI) would be inducing a cycle of breaks constantly forming and repairing over the exposure period. Arguing against this possibility is the reduced HR repair response due the observed effects on Rad51. Of course, it is possible that a switch to NHEJ repair might offset the loss of HR repair, but currently, there is no evidence that such an outcome occurs.

An alternative possibility is that some breaks form and are sensed by the repair pathway, but in the absence of an effective HR Rad51 response, these breaks escape cell cycle arrest and progress to become chromosomal aberrations. This outcome is consistent with our observations that Mre11 and ATM foci increase with dose and time whereas the Rad51 foci decline over time at each dose, indicating the breaks are sensed and the repair signal transduced, but the effector arm cannot effectively respond. It is also consistent with our observations that chromosomal aberrations also increase with dose and time.

Of course, it is possible both outcomes are occurring with the switch to NHEJ repair occurring at later time points, possibly explaining the spike at 96 h. The Rad51 response does not return to baseline until 96 h. Perhaps, there is sufficient Rad51 to allow HR repair for the first 72 h of exposure. Then, at 96 h, there is a loss of HR repair causing an increase in breaks, leading to a switch to NHEJ repair that reduces the breaks back to the equilibrium seen at 120 h.

A third possibility relates to how the breaks form and arrest in the cell cycle. For both particulate and soluble Cr(VI), the breaks are produced indirectly and only in the G2 phase of the cell cycle (Xie et al., 2005; Ha et al., 2004). The underlying cause has been attributed to the collapse of a stalled replication fork leading to the double strand break, but could also be due to an unrepaired single strand break entering S or G2 and being converted to a DNA double strand break, as both particulate and soluble Cr(VI) cause DNA double strand breaks (Wise et al., 2008). Regardless of which mechanism forms the breaks, it could be that the cells arrest in the cell cycle for a protracted period of time (i.e., several days) and there is a diminution of breaks as arrested cells might not allow for new breaks to form. However, there are no data showing that Cr(VI) can induce an individual cell to remain arrested for several days to support this possibility. The current analyses are restricted to population measurements showing some arrest continues, but not whether it is cells entering and exiting the arrest or the same cells simply remaining arrested. Further arguing against this possibility is our observations of concentration-dependent response. If the cells were to arrest and not proceed due to the damage, then one would expect this arresting effect to be greater in higher concentrations as more damage would presumably equal more arrest. Thus, one would expect in this scenario to see less damage at higher doses due to the increased arrest, when in fact we see more damage at higher concentrations. Furthermore, chromosomal aberrations are considered indirect markers of DNA double strand breaks and this endpoint increases with dose and time suggesting there are plenty of breaks making in through the arrest.

In sum, previous studies showed loss of DNA double strand break repair was required for particulate Cr(VI)-induced neoplastic transformation. This study is the first to show particulate Cr(VI) alters DNA double strand break repair in human lung cells. In particular, longer particulate Cr(VI) exposures inhibit the Rad51 response. Thus, we propose a new aspect to the carcinogenic mechanism for Cr(VI). Specifically, after acute exposures (i.e., <24 h) Cr(VI) induces a G2-dependent DNA double strand breaks which cells address with the canonical HR response at 96 h. The Rad51 response does not return to baseline until 96 h. Perhaps, there is sufficient Rad51 to allow HR repair for the first 72 h of exposure. Then, at 96 h, there is a loss of HR repair causing an increase in breaks, leading to a switch to NHEJ repair that reduces the breaks back to the equilibrium seen at 120 h.
SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

National Institute of Environmental Health Sciences (ES016893 to J.P.W.); National Aeronautics and Space Administration (NASA) (ACD FSB–2009 to J.P.W.); Maine Center for Toxicology and Environmental Health. Funding for open access charge: National Institutes of Health (ES016893 to J.P.W.); NASA (ACD FSB–2009 to J.P.W.).

ACKNOWLEDGMENTS

We thank Geron Corporation for the use of the hTERT materials. We would like to thank Christy Gianios and Shouping Huang for administrative and technical assistance. Conflict of interest: The author acknowledges that he has grants from NIH and NASA to do research in this area; the funding organizations do not have control over the resulting publication.

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