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Prolonged exposure to particulate chromate inhibits RAD51 nuclear import mediator proteins

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ABSTRACT

Particulate hexavalent chromium (Cr(VI)) is a human lung carcinogen and a human health concern. The induction of structural chromosome instability is considered to be a driving mechanism of Cr(VI)-induced carcinogenesis. Homologous recombination repair protects against Cr(VI)-induced chromosome damage, due to its highly accurate repair of Cr(VI)-induced DNA double strand breaks. However, recent studies demonstrate Cr(VI) inhibits homologous recombination repair through the misregulation of RAD51. RAD51 is an essential protein in HR repair that facilitates the search for a homologous sequence. Recent studies show prolonged Cr(VI) exposure prevents proper RAD51 subcellular localization, causing it to accumulate in the cytoplasm. Since nuclear import of RAD51 is crucial to its function, this study investigated the effect of Cr(VI) on the RAD51 nuclear import mediators, RAD51C and BRCA2. We show acute (24 h) Cr(VI) exposure induces the proper localization of RAD51C and BRCA2. In contrast, prolonged (120 h) exposure increased the cytoplasmic localization of both proteins, although RAD51C localization was more severely impaired. These results correlate temporally with the previously reported Cr(VI)-induced RAD51 cytoplasmic accumulation. In addition, we found Cr(VI) does not inhibit interaction between RAD51 and its nuclear import mediators. Altogether, our results suggest prolonged Cr(VI) exposure inhibits the nuclear import of RAD51C, and to a lesser extent, BRCA2, which results in the cytoplasmic accumulation of RAD51. Cr(VI)-induced inhibition of nuclear import may play a key role in its carcinogenic mechanism since the nuclear import of many tumor suppressor proteins and DNA repair proteins is crucial to their function.

1. Introduction

Particulate hexavalent chromium (Cr(VI)) is an established human lung carcinogen (IARC, 1990). Epidemiological studies of chromate workers published since 1891 have documented the occurrence of Cr(VI)-induced respiratory cancers (Davies et al., 1991; Newman, 1890; Rosenman and Stanbury, 1996). A wealth of data from animal and cell culture studies further supports the carcinogenic potential of this heavy metal (Balansky et al., 2000; Costa et al., 2010; Levy and Venitt, 1986; Xie et al., 2007). Due to its carcinogenicity and frequent occupational and environmental exposure, particulate Cr(VI) is human health concern (IARC, 1990; OSHA, 2015).

A recent study showed Cr(VI) inhibits the high fidelity DNA repair pathway, homologous recombination (HR) in human lung cells (Browning et al., 2016). While HR repair is active after acute (24 h) Cr(VI) exposure, this repair pathway is inhibited by prolonged (120 h) exposure (Browning et al., 2016). Since HR repair is protective against Cr(VI)-induced chromosome damage (Stackpole et al., 2007), the inhibition of this repair pathway is an important component in the carcinogenetic mechanism of Cr(VI). This is exemplified by the fact that the Cr(VI)-induced inhibition of HR repair correlates with increased structural chromosome damage (Browning et al., 2016; Qin et al., 2014).

We have previously shown prolonged Cr(VI) exposure specifically targets the central HR repair protein, RAD51, by inhibiting protein levels and its proper localization to the nucleus (Browning et al., 2016; Qin et al., 2014). Like many DNA repair proteins and tumor suppressors, RAD51 nuclear transport is crucial to its function (Essers et al., 2002; Fabbro and Henderson, 2003; Gildemeister et al., 2009). Nuclear RAD51 levels are tightly regulated to prevent random recombination events from occurring. Upon DNA damage, RAD51 is transported into the nucleus (Essers et al., 2002; Gildemeister et al., 2009). Thus, Cr(VI)-induced RAD51 subcellular mislocalization is an important component of the mechanism of Cr(VI)-induced HR repair inhibition.

Previous studies show BRCA2 and RAD51C both import RAD51 into the nucleus independently of each other (Gildemeister et al., 2009; Jeyasekharan et al., 2013). We previously observed Cr(VI)-induced RAD51 mislocalization to the cytoplasm corresponded temporally

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with decreased RAD51C foci formation (Browning et al., 2016). This outcome suggests impaired RAD51 nuclear import may be a factor, for Cr(VI)-induced RAD51 cytoplasmic accumulation. Thus, the goal of this study is to investigate the effect of prolonged Cr(VI) exposure on the RAD51 nuclear import mediator proteins, RAD51C and BRCA2, focusing on their interaction with RAD51 and their subcellular localization.

2. Materials and methods

2.1. Chemicals and reagents

DMEM and Ham's F12 50:50 mixture and GlutaGRO (L-alanyl-L-glutamine solution) were purchaed from Mediatech Inc. (Herndon, VA). Cosmic Calf Serum was purchased from Hyclone (Logan, UT). Dulbecco's phosphate buffered saline (PBS), goat serum, HEPES, penicillin/streptomycin, sodium pyruvate, trypsin/EDTA, Prolong Gold Antifade Reagent with DAPI and Alexa Fluor 488 were purchased from Life Technologies (Grand Island, NY). Plasticware was purchased from BD Biosciences (Franklin Lakes, NJ). Nunc Lab Tek II glass and permanox chamber slides and attachment factor protein were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). FNC coating mix was purchased from AthenaFS (Baltimore, MD). Zinc chromate (CAS#13530-65-9) was purchased from Alfa Aesar (A18178, Ward Hill, MA) and Pfaltz and Bauer (Z00277, Waterbury, CT). 4% paraformaldehyde in PBS was purchased by Alfa Aesar (Ward Hill, MA). Triton X-100, Igepal and Duolink *in situ* orange starter kit mouse/rabbit (DUO92102) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride, 2-mercaptoethanol and bovine serum albumin were purchased from EMD Millipore (Billerica, MA). Potassium chloride, sodium dodecyl sulfate and methanol were purchased from VWR (Randor, PA). Cleland Reagent (DTT), PMSF, Tris, glycine and Tween-20 were purchased from Amresco LLC (Solon, OH). PhosSTOP phosphatase inhibitor tablets and complete ULTRA protease inhibitor tablets were purchesed from Roche Diagnostics Corporation (Indianapolis, IN). PVDF membrane and Bradford assay kit were purchased from BioRad (Hercules, CA) and Odyssey blocking buffer was purchased from LiCor (Lincoln, NE).

2.2. Cell culture

We used WTHBF-6 cells, an hTERT immortalized clonal cell line derived from human bronchial fibroblasts, to investigate the effect of Cr(VI) on DNA repair proteins. 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). To study the RAD51C gene, we also used the model Chinese hamster lung fibroblast cell lines: V79, irs3 and irs3#6. The irs3 cell line, derived from the parental V79 cells, expresses a mutated RAD51C gene resulting in undetectable levels of RAD51C protein. The RAD51C cDNA-complimented cell line, irs3#6 cells, express RAD51C protein levels comparable to the parental cell line, V79. Details and validation of these cells have been previously published (French et al., 2002; Stackpole et al., 2007). All cells were cultured as adherent monolayers in DMEM/F12 50:50 mixture, supplemented with 15% cosmic calf serum, 1% L-alanyl-L-glutamine, 1% penicillin/streptomycin, and 0.1 mM sodium pyruvate. Cells were maintained in a 5% CO_2-humidified environment at 37 °C.

2.3. Treatment with particulate Cr(VI) compound

Zinc chromate was administered as a suspension of particles in cold, sterile water as previously described (Xie et al., 2009). After seeding, cells were allowed to reenter logarithmic growth before treatment. Cells were treated for 24, 72 and 120 h with concentrations of 0.1–0.3 μg/cm^2 zinc chromate. Zinc chromate induces a time and concentration dependent increase in cytotoxicity, but plenty of cells survive and proliferate after 120 h exposure to these concentrations (Holmes et al., 2010). We selected 24 h and ≥72 h to represent acute and prolonged Cr(VI) exposure, respectively, because RAD51 and HR repair activity differ in human lung cells after 24 and ≥72 h exposures to 0.1–0.3 μg/cm^2 zinc chromate (Browning et al., 2016; Qin et al., 2014).

2.4. Immunofluorescence

Immunofluorescence staining was conducted as previously described with minor alterations (Xie et al., 2005). Briefly, WTHBF-6 cells were seeded on glass chamber slides coated with FNC while the Chinese hamster cell lines were seeded on permanox chamber slides coated with attachment factor. After zinc chromate treatment, cells were fixed with 4% paraformaldehyde for 10 mins, permeabilized with 0.2% Triton X-100 for 5 mins and blocked with 10% goat serum and 5% BSA in PBS for 1 h. For BRCA2 staining, cells were incubated with 0.5% Triton X-100 for 5 mins, fixed with 4% paraformaldehyde and 0.5% Triton X-100 for 15 mins and blocked with 2.5% BSA. Cells were then incubated with anti-RAD51C (abcam ab72063; 1:500) or anti-BRCA2 (Genetex GTX70121; 1:50) antibodies at 4 °C overnight, washed with PBS and incubated with Alexa Fluor 488 (1:3000) for 1 h. Cells were washed with PBS and coverslipped with DAPI. Images of 50 cells per concentration/timepoint were obtained by confocal microscopy. Whole cell and nuclear intensities were measured as integrated intensity using Image J (http://imagej.nih.gov/ij/). Cytoplasmic integrated intensity was determined by subtracting the nuclear intensity value from the whole cell intensity value. Cells with a cytoplasmic intensity greater than the mean intensity + 1 SEM (standard error of the mean) of control cells were considered positive for cytoplasmic accumulation.

2.5. Proximity ligation assay (PLA)

Cells were seeded in 8 well permanox slides, allowed to reenter logarithmic growth and treated with zinc chromate. After treatment, cells were fixed with 4% paraformaldehyde with 0.5% Triton X for 15 min at 4 °C and permeabilized with 0.2% Triton X-100 for 5 min. RAD51/RAD51C and RAD51/BRCA2 wells were blocked for 1 h with 10% goat serum and 5% BSA in PBS or 2.5% BSA in PBS, respectively. RAD51/RAD51C wells were incubated with anti-RAD51 (GeneTex GTX70230; 1:100) and anti-RAD51C (abcam ab72063; 1:500) while RAD51/BRCA2 wells were incubated with anti-RAD51 (Santa Cruz sc-8349; 1:200) and anti-BRCA2 (Genetex GTX70121; 1:50) at 4 °C overnight.

To prepare for the PLA reaction, slides were washed with 1× PBS and the chamber removed, leaving behind the silicon seal. The cells underwent consecutive incubations with; the PLA probes (diluted 1:5) for 1 h; ligation stock and ligase (diluted 1:5 and 1:40) for 30 min; amplification stock and polymerase (diluted 1:5 and 1:80) for 2 h. Slides were washed in 1× Duolink wash buffer A between incubations and all incubations were conducted at 37 °C in a humidity chamber. After the last incubation, slides were washed in 1× Duolink wash buffer B, dried, coverslipped with Duolink DAPI and stored at −20 °C. A negative control, containing no primary antibodies was used to demonstrate that no background foci formed as a result of the PLA components.

For each protein combination, images of 50 cells per concentration/timepoint were obtained by confocal microscopy. Nikon Elements AR software was used to count the number of foci in each cell and nuclei. The number of cytoplasmic foci was determined by subtracting the number of nuclear foci from the number of whole cell foci.

2.6. Western blot

Cells were plated into 100 mm dishes and treated with zinc chromate. After treatment, nuclear extracts were prepared according to our published methods (Qin et al., 2014). Protein concentration was determined with a Bradford assay and 10 μg protein were resolved on 12% Bis-Tris SDS-PAGE gels and transferred to PVDF membranes.