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Assessment of genotoxic potential of Cr(VI) in the mouse duodenum: An in silico comparison with mutagenic and nonmutagenic carcinogens across tissues

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ABSTRACT

In vitro studies on hexavalent chromium [Cr(VI)] indicate that reduced forms of this metal can interact with DNA and cause mutations. Recently, Cr(VI) was shown to induce intestinal tumors in mice; however, Cr(VI) elicited redox changes, cytotoxicity and hyperplasia – suggesting involvement of tissue injury rather than direct mutagenesis. Moreover, toxicogenomic analyses indicated limited evidence for DNA damage responses. Herein, we extend these toxicogenomic analyses by comparing the gene expression patterns elicited by Cr(VI) with those of four mutagenic and four nonmutagenic carcinogens. To date, toxicogenomic profiles for mutagenic and nonmutagenic duodenal carcinogens do not exist, thus duodenal gene changes in mice were compared to those elicited by hepatocarcinogens. Specifically, duodenal gene changes in mice following exposure to Cr(VI) in drinking water were compared to hepatic gene changes previously identified as potentially discriminating mutagenic and nonmutagenic hepatocarcinogens. Using multivariate statistical analyses (including logistic regression classification), the Cr(VI) gene responses clustered apart from mutagenic carcinogens and closely with nonmutagenic carcinogens. These findings are consistent with other intestinal data supporting a nonmutagenic mode of action (MOA). These findings may be useful as part of a full weight of evidence MOA evaluation for Cr(VI)-induced intestinal carcinogenesis. Limitations to this analysis will also be discussed.

1. Introduction

Inhalation of hexavalent chromium [Cr(VI)] has long been recognized to pose a carcinogenic risk to the lung (IARC, 1990). Oral exposure to Cr(VI) at environmentally relevant levels has been widely considered not to pose a cancer risk due to reduction of Cr(VI) to poorly absorbed Cr(III) by bodily fluids and cellular constituents (De Flora et al., 1997; Proctor et al., 2002; US EPA, 1991). However, chronic exposure to very high levels of Cr(VI) in drinking water resulted in intestinal neoplasms in mice in a recent 2-year bioassay (NTP, 2008). Notably, the carcinogenic Cr(VI) concentrations in the drinking water were bright yellow and were associated with reduced water intake due to unpalatability (NTP, 2008; Thompson et al., 2011b). Considering that the intestinal carcinogenesis in mice occurred at unusually high Cr(VI) concentrations, it is critical to understand the mode of action (MOA) of the intestinal tumors in mice because it informs the relevance of these tumors to humans as well as the low-dose extrapolation methods employed for the derivation of Cr(VI) toxicity criteria in various media (e.g. drinking water). To this end, a comprehensive research program was conducted to gather critical data needed to inform the MOA underlying Cr(VI)-induced intestinal carcinogenesis (Kopec et al., 2012a; Thompson et al., 2011a,b).

An important consideration in these studies is whether Cr(VI) acts via a mutagenic or nonmutagenic MOA in the small intestine. Mutagens interact directly with DNA and are generally thought to exhibit a non-thresholded dose–response,[1] whereas nonmutagenic (i.e. indirect) genotoxic carcinogens and nongenotoxic carcinogens exhibit thresholded behavior (Bolt et al., 2004; Eastmond, 2008). As part of our research into the MOA of Cr(VI)-induced intestinal carcinogenesis, *in vivo* micronucleus formation and *k-ras* mutations

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[1] However, according to US EPA (2005a), "Special attention is important when the data support a nonlinear mode of action but there is also a suggestion of mutagenicity. Depending on the strength of the suggestion of mutagenicity, the assessment may justify a conclusion that mutagenicity is not operative at low doses and focus on a nonlinear approach…".

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were assessed in duodenal tissue sections of mice exposed to Cr(VI) up to 90 days, and were found to be negative (Harris et al., 2012; O'Brien et al., in preparation). Toxicogenomic evaluation of responses to Cr(VI) in the mouse small intestine indicated activation of Nrf2 signaling at relatively low exposure concentrations (Kopec et al., 2012a), consistent with clear alteration in cellular redox status in similarly treated mice (Thompson et al., 2011b). Several genes involved in DNA repair were elevated by day 8 of exposure to carcinogenic concentrations of Cr(VI), and functional analysis indicated enrichment of DNA repair pathways at the highest Cr(VI) concentrations (Kopec et al., 2012a). Beyond transcriptional and functional analyses, it is of interest to scientists and risk assessors to examine whether the genomic signature/profile of Cr(VI) is similar to that of known mutagens. Due in part to the low incidence of cancer in the small intestine (Greaves, 2007), there are insufficient toxicogenomic profile data from the rodent small intestine with which to compare the duodenal toxicogenomic data of mice treated with Cr(VI). There are, however, toxicogenomic comparisons for mutagenic and nonmutagenic carcinogens in rodent liver.

Ellinger-Ziegelbauer et al. (2005) exposed rats to carcinogenic concentrations of eight hepatocarcinogens (four mutagenic and four nonmutagenic) for up to 2 weeks, and identified a subset of genes that were useful for distinguishing between mutagenic and nonmutagenic hepatic carcinogens. In the absence of comparable intestinal data, we compared the differential expression of genes in the mouse duodenum following Cr(VI) treatment with the differential expression reported by Ellinger-Ziegelbauer and colleagues. To facilitate the comparison of the differential expression of genes across nine chemicals, we used data reduction techniques (e.g. principal components analysis, PCA) and multivariate analyses to make unbiased evaluation of whether Cr(VI) was similar to the mutagenic or nonmutagenic carcinogens. The results indicate that the expression pattern induced by Cr(VI) more closely follows that latter. These findings, notwithstanding the limitations discussed herein, may be useful as part of a weight of evidence to evaluate the MOA for Cr(VI)-induced intestinal carcinogenesis.

2. Material and methods

2.1. Animal treatments and tissue preparation

Test substance, animal husbandry, and study design have been described in detail elsewhere (Thompson et al., 2011b). Briefly, female B6C3F1 mice (Charles Rivers Laboratories International, Inc.) were provided *ad libitum* access to Cr(VI), as sodium dichromate dihydrate (SDD), in drinking water at concentrations ranging from 0.3–520 mg/L. After 7 and 90 days of exposure (referred to herein respectively as day 8 and 91), animals were euthanized using CO_2. For toxicogenomic analyses, duodenal samples were scraped and processed as described previously (Kopec et al., 2012a).

2.2. Microarray analysis of Cr(VI) data

Details on mouse 4x44 K Agilent whole-genome oligonucleotide microarrays and data analysis for SDD-elicited duodenal gene expression at day 8 and 91 are described in Kopec et al. (2012a). In brief, total RNA was isolated according to the manufacturer's protocol with an additional acid phenol:chloroform extraction, resuspended in RNA storage solution (Ambion Inc., Austin, TX), quantified (A_{260}), and quality was assessed by evaluation of the A_{260}/A_{280} ratio and by visual inspection of 1 μg total RNA on a denaturing gel. Dose-dependent changes in gene expression were examined using mouse 4×44 K Agilent whole-genome oligonucleotide microarrays (Agilent Technologies, Inc., Santa Clara, CA). All experiments were performed with three biological replicates and independent labeling of each sample (Cy3 and Cy5, and dye swap) for every dose group at each time point. Microarray data were normalized using a semi-parametric approach (Eckel et al., 2004, 2005).

2.3. Gene expression data selection for comparisons

These Cr(VI) gene expression data were compared to previously published gene expression data for four genotoxic and four nongenotoxic hepatic carcinogens (Ellinger-Ziegelbauer et al., 2005). The genotoxic carcinogens were 2-nitrofluorene (2-NF), dimethylnitrosamine (DMN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and aflatoxin B1 (AB1); the nongenotoxic carcinogens were methapyrilene (MPy), diethylstilbestrol (DES), Wy-14643 (WY), and piperonylbutoxide (PBO). Importantly, the four "genotoxic" hepatic carcinogens were characterized as inducing DNA modification and causing mutations and physical distortion of DNA (Ellinger-Ziegelbauer et al., 2004, 2005), and have been characterized as mutagens by the U.S. EPA (US EPA, 2005b; US EPA, 2007). Thus, we will use the terminology mutagenic/nonmutagenic as opposed to the genotoxic/nongenotoxic terminology used by Ellinger-Ziegelbauer and colleagues. Moreover, the term genotoxic is somewhat ambiguous. For example, WY can induce oxidative DNA damage (i.e. genotoxicity), yet many scientists consider carcinogens that work primarily through oxidative stress and oxidative damage as nongenotoxic (Ellinger-Ziegelbauer et al., 2005; Klaunig et al., 1998).

Treatment of rats with these eight carcinogens resulted in significant expression of 651 probe sets, corresponding to 477 nonredundant genes, as measured by Affymetrix RG U34A arrays, which were further grouped into 23 toxicological categories (Ellinger-Ziegelbauer et al., 2005). Seven categories (comprised of 139 genes) were discussed in greater detail by Ellinger-Ziegelbauer and colleagues: *oxidative stress/DNA response* (13), *oxidative stress/protein damage response* (25), *oxidative stress response* (13), *stress response* (9), *regeneration* (34), *cell survival and/or proliferation* (25), and *cell cycle progression* (20). Several (but not all) of the genes in these categories were differentially expression by the two classes of carcinogens. Gene expression data for all 477 genes were obtained from the Supplementary Material in Ellinger-Ziegelbauer et al. (2005). Data were available for 1, 3, 7 and 14 days of exposure; however, only the 7 day exposure data were averaged (3 replicates for DMN, NNK, AB1, MPy, DES, Wy, PBO and 2 replicates for 2-NF) in order to obtain a single day 8 expression value for each of the 139 genes for each of the 8 carcinogens.

The treatment doses employed in Ellinger-Ziegelbauer et al. (2005) were concentrations known to be carcinogenic to rats in longer-term bioassays; moreover, the doses also caused histopathological changes in the course of their short-term study. For consistency, we therefore selected the 520 mg/L SDD (182 mg/L Cr(VI)) treatment group because it is carcinogenic in a 2-year bioassay (NTP, 2008), and elicited histopathological lesions in the mouse duodenum at day 8 (Thompson et al., 2011b).

To allow for direct comparison between our data and those of Ellinger-Ziegelbauer et al. (2005), 651 significant Affymetrix rat probes from Ellinger-Ziegelbauer et al. were converted to unique HomoloGeneIDs using Database for Annotation, Visualization, and Integrated Discovery and mapped to the mouse whole-genome 4×44 K Agilent array. Of the 477 unique genes, orthologous mapping identified 395 (82%) mouse genes for which Cr(VI)-elicited gene expression changes at 520 mg/L SDD were available. Of the 139 unique genes in the 7 aforementioned categories, orthologous mapping identified 116 (83%) mouse genes for which Cr(VI)-elicited gene expression changes at 520 mg/L SDD were available.