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Assessment of K-*Ras* mutant frequency and micronucleus incidence in the mouse duodenum following 90-days of exposure to Cr(VI) in drinking water

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ABSTRACT

Chronic exposure to high concentrations of hexavalent chromium [Cr(VI)] as sodium dichromate dihydrate (SDD) in drinking water induces duodenal tumors in mice, but the mode of action (MOA) for these tumors has been a subject of scientific debate. To evaluate the tumor-site-specific genotoxicity and cytotoxicity of SDD in the mouse small intestine, tissue pathology and cytogenetic damage were evaluated in duodenal crypt and villus enterocytes from B6C3F$_1$ mice exposed to 0.3–520 mg/L SDD in drinking water for 7 and 90 days. Allele-competitive blocker PCR (ACB-PCR) was used to investigate the induction of a sensitive, tumor-relevant mutation, specifically *in vivo* K-*Ras* codon 12 GAT mutation, in scraped duodenal epithelium following 90 days of drinking water exposure. Cytotoxicity was evident in the villus as disruption of cellular arrangement, desquamation, nuclear atypia and blunting. Following 90 days of treatment, aberrant nuclei, occurring primarily at villi tips, were significantly increased at ≥60 mg/L SDD. However, in the crypt compartment, there were no dose-related effects on mitotic and apoptotic indices or the formation of aberrant nuclei indicating that Cr(VI)-induced cytotoxicity was limited to the villi. Cr(VI) caused a dose-dependent proliferative response in the duodenal crypt as evidenced by an increase in crypt area and increased number of crypt enterocytes. Spontaneous K-*Ras* codon 12 GAT mutations in untreated mice were higher than expected, in the range of 10^{-2} to 10^{-3}; however no treatment-related trend in the K-*Ras* codon 12 GAT mutation was observed. The high spontaneous background K-*Ras* mutant frequency and Cr(VI) dose-related increases in crypt enterocyte proliferation, without dose-related increase in K-*Ras* mutant frequency, micronuclei formation, or change in mitotic or apoptotic indices, are consistent with a lack of genotoxicity in the crypt compartment, and a MOA involving accumulation of mutations late in carcinogenesis as a consequence of sustained regenerative proliferation.

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1. Introduction

Chronic exposure to high concentrations of hexavalent chromium [Cr(VI)], as sodium dihydrate dichromate (SDD), in drinking water was recently shown to cause small intestinal tumors in mice [1,2]. These tumors occurred at concentrations that are orders of magnitude greater than typical environmental exposures to Cr(VI). Specifically, mouse duodenal tumors were observed at 57–516 mg/L SDD or ~20–180 mg/L Cr(VI), whereas environmental exposures for humans are common at drinking water concentrations of 0.001–0.005 mg/L Cr(VI) [3]. Ingestion of Cr(VI) has generally not been considered carcinogenic at environmentally relevant exposures because most Cr(VI) is reduced to trivalent

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chromium [Cr(III)] in the acidic conditions of the stomach thereby limiting uptake [4]. However, because Cr(VI) is genotoxic and thus may potentially act by a mutagenic mode of action (MOA) to cause mouse intestinal tumors [5,6], the potential for Cr(VI) to pose a cancer risk in the low dose range has been the subject of significant scientific debate and study [3,7]. Several MOAs have been proposed for Cr(VI)-induced small intestinal carcinogenesis [3,5,6]. If mutation is an early event for the MOA, then mutations in target tissues are expected to increase as a function of dose, and detected at lower doses and prior to the onset of pre-neoplastic and neoplastic lesions. Considering that environmental exposure to low levels of Cr(VI) in drinking is widespread [8–11], whether Cr(VI) acts by a mutagenic MOA and poses a potential cancer risk at low exposures are important questions for protecting public health.

The analysis of specific hotspot oncogene mutations is a powerful approach for understanding MOA. Further, *in vivo* mutational analysis in the tissues where tumors occur is the highest tier of evidence for determining whether a chemical operates *via* a mutagenic MOA as delineated in the U.S. EPA's draft Framework for Determining a Mutagenic Mode of Action for Carcinogenicity [12]. Studies using model mutagenic carcinogens (aristolochic acid, azoxymethane, benzo[a]pyrene, N-hydroxy-acetylaminofluorene, and simulated solar light) have shown that allele-specific competitive blocker-PCR (ACB-PCR) is sufficiently sensitive to detect the early, dose-related increases in mutations that lead to tumor formation. In addition, several studies show that K-*Ras* codon 12 GGT to GAT mutation, a mutation frequently detected in normal/control tissues (human/rodents), may be amplified during carcinogenesis, even when the mutational specificity of the mutagen is other than the G to A mutation being measured [13,14]. Both K-*Ras* codon 12 GGT to GAT and GGT to GTT mutations were recently shown to occur following 4 weeks of inhalation exposure to ethylene oxide in Big Blue B6C3F1 mouse lung tissue [15]. Because ethylene oxide induces oxidative stress, G to T mutation was hypothesized to occur, but the K-*Ras* codon 12 GAT mutation was the more sensitive endpoint, more than K-*Ras* codon 12 GTT or cII neutral reporter gene mutations [15]. These results are interpreted as meaning that amplification of spontaneous K-*Ras* codon 12 GAT mutation, potentially through cooperative interactions with other chemically induced mutations, can serve as a sensitive, generic reporter of the early effects of a carcinogen.

Although mutational specificity was not reported by the National Toxicology Program (NTP) for the mouse small intestinal tumors, K-*Ras* may be the mutation target for mouse small intestinal tumors induced by Cr(VI). It is among the most frequently activated oncogenes with mutations at codons 12, 13, and/or 61 found in mammalian tumors [16]. K-*Ras*-mediated signaling plays a key role in controlling cell cycle progression, growth, migration, cytoskeletal changes, proliferation, differentiation, and apoptosis [16,17]. Although very little is known about the potential role that K-*Ras* mutations play in the formation of relatively rare duodenal tumors in rodents or humans, codon 12 GGT to GAT mutation is one of the most commonly reported mutations in human intestinal cancers [18–22]. In addition, K-*Ras* mutation has been shown to contribute to small intestine tumorigenesis in mice and is mutated early in carcinogenesis [23,24]. Based on these facts, K-*Ras* is considered a potential target oncogene, in addition to being a non-specific reporter gene, for intestinal carcinogenesis.

The objective of this study was to assess whether mutagenesis is an early key event in the MOA for Cr(VI)-induced duodenal carcinogenesis. Using a multifaceted approach, we investigated genotoxicity and mutagenicity in mouse duodenal epithelium following oral exposures to SDD for 90 days under conditions similar to those employed in the NTP 2-year cancer drinking water bioassay (0, 14, 60, 170 and 520 mg/L SDD) and at two lower exposure concentrations (0.3 and 4 mg/L SDD) that are more representative of possible human exposure. Considering the unique structure of the small intestine with non-proliferating villi and proliferating crypt compartments (Fig. 1A), duodenal tissues were examined for evidence of aberrant nuclei (*e.g.*, micronuclei) in the crypt and villus regions. Taken together, assessment of the effects of Cr(VI) on the crypt compartment and on the frequency of K-*Ras* codon 12 GAT mutation greatly improves our understanding of the MOA for the small intestinal tumors observed in mice following lifetime exposure to Cr(VI) in drinking water.

2. Materials and methods

2.1. Animals and tissues

Test substance, animal husbandry, and study design have been described in detail elsewhere [7]. Briefly, female B6C3F$_1$ mice (Charles Rivers Laboratories International, Inc., Raleigh, NC) were provided *ad libitum* access to SDD (Sigma–Aldrich, Inc. Milwaukee, WI) in drinking water at concentrations ranging from 0.3 to 520 mg/L for 7 or 90 days (effects are referred to herein as occurring on days 8 and 91). At the time of sacrifice, intestinal sections were flushed with ice-cold phosphate buffered saline. The duodena were cut longitudinally, and the epithelium was scraped, and stored at −80 °C.

For assessment of tissue histopathology, aberrant nuclei and mitotic figures, duodenal sections were fixed in 10% neutral buffered formalin, embedded in paraffin for transverse sectioning and sectioned at approximately 5 μm. All procedures were carried out with the approval of the Institutional Animal Care and Use Committee at Southern Research Institute.

2.2. Assessment of crypt area, aberrant nuclei and mitotic figures in mouse duodenum

Paraffin-embedded duodenal sections (3 sections per mouse) were stained for DNA using Feulgen's stain and analyzed by Experimental Pathology Laboratories, Inc. (EPL®; Sterling, VA). Unless otherwise stated, image analysis procedures were performed according to methods described in the EPL standard operating procedures. Using the 40× microscope objective and the Virtual Slice module of the Stereo Investigator software, a montage image (multiple high-resolution images stitched together) was obtained. Using Image-Pro® Plus (IPP; v7.0, Media Cybernetics, Silver Spring, MD) software, the total mucosal and villous surface areas were outlined manually and the internal borders of these areas were determined automatically by the software's "Count/Size" color segmentation tool and user-defined colorimetric criteria. The crypt area was calculated by subtracting the villous area from the total mucosal area.

Mitotic and apoptotic cells were counted in fully intact crypts in order to compute mitotic and apoptotic indices. Furthermore, karyorrhectic nuclei, micronuclei (MN) and apoptotic nuclei were counted in both the villus and crypt compartments of three slides obtained from each animal per dose group (4–5 mice in each of 7 dose groups). Digital images were randomized prior to the cell counting, and persons performing the counts were unaware of the treatment group status of individual animals. Using IPP software, aberrant nuclei in the digital image were counted by marking each nucleus manually. Identification criteria for aberrant nuclei (*i.e.*, micronuclei, apoptotic nuclei, and karyorrhectic nuclei) were consistent with that of Goldberg et al. [25]. Specifically, apoptotic cells were characterized by nuclei that had a smudged, heterochromatic appearance, and a discrete rounding of the cell cytoplasm was often evident. Conversely, distinctly visible chromosomal components were apparent in cells with mitotic figures. Each MN consisted of a single dense, ovoid to spherical body that was located adjacent to a normal nucleus within the cytoplasm of the same cell. Karyorrhectic nuclei were fragmented into small, unequally sized, dense spherical bodies, and the cytoplasmic margins of such cells were often indistinct.

Duodenal tissue sections stained with hematoxylin and eosin (H&E) were previously prepared as described in Thompson et al. [7] for microscopic examination. Tissue sections were assessed and scored for stratification, desquamation, presence of lymphocytes in the lamina propria, nuclear atypia and the ratio of villous to crypt length (Appendix A for methods and scoring results).

2.3. DNA isolation and amplification of K-Ras from mouse duodenum samples

For DNA isolation, previously reported methods [26] were followed. A region surrounding the K-*Ras* gene was amplified from 1 μg of digested genomic DNA (~3×10^5 copies of a single-copy nuclear genome) per 200 μl PCR reaction containing: 20 μl 10× Pfu reaction buffer, 16 μl of 2.5 mM dNTP, 4 μl PfuUltra™ DNA polymerase and 4 μl (10 μM) each of primer (forward primer, 5′-TGGCTGCCGTCCTTTACAA-3′ and reverse primer, 5′-GGCCTGCTGAAAATGACTGAGTATAAACTTGT-3′). Cycling conditions were: 2 min at 94 °C, 28 cycles of 1 min at 94 °C, 2 min at 58 °C, 1 min at 72 °C followed by a 7 min final extension at 72 °C. The PCR product (170 bp) was concentrated by centrifugation under vacuum to ~50 μl and resolved on a 1.5% TAE agarose gel,