Review Article

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Carcinogenicity of hexavalent chromium

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Hexavalent chromium (Cr(VI)), a commonly used industrial metal, is a well known human lung carcinogen. Epidemiology and animal studies suggest that the particulate Cr(VI) compounds, specifically the water insoluble compounds, are the more potent carcinogens; however, the carcinogenic mechanism remains unknown. Here we summarize recent Cr(VI)-induced human tumour, *in vivo*, cell culture and *in vitro* studies and put the data into context with three major paradigms of carcinogenesis: multistage carcinogenesis, genomic instability, and epigenetic modifications. Based on these studies, we propose a mechanism for chromate carcinogenesis that is primarily driven by the genomic instability paradigm.

Key words Chromate - chromium - chromosome instability - epigenetic changes - genomic instability - hexavalent chromium - lead chromate - mutations - zinc chromate

Introduction/overview

For more than 100 years, numerous epidemiological studies have been performed on workers exposed to hexavalent chromium (Cr(VI)) to determine its carcinogenicity. Major studies pinpointing Cr(VI) as a human lung carcinogen have been performed on workers involved in chromate production, chromate pigment production and chromium plating. Chromate production involves combining trivalent chromite ore with soda ash and sometimes lime and heating the mixture to high temperatures causing the chromite to oxidize to soluble sodium chromate. If lime is used in the production process, insoluble calcium chromate is also produced. The first epidemiology study on chromate production workers was performed in 1948. Machle and Gregorius showed that 21.8 per cent of the chromate workers deaths were due to respiratory cancer compared to only 1.4 per cent in their reference population[1]. Large studies on chromate production workers have also been performed in Baltimore, Maryland; Plainsville, Ohio; and Great Britain[2,3]. Altogether, these studies indicate that these workers have about a 2-80 fold increased relative risk of developing lung cancer and that exposure to Cr(VI) and not Cr(III) was responsible for this effect[2-4]. Lung cancer risk also increased with exposure time. These studies indicate that insoluble calcium chromate is more carcinogenic than soluble sodium chromate because lung cancer rates decreased after factories changed to a low or no lime production process[2].

Chromate pigment production involves reacting sodium chromate with zinc or lead to produce insoluble zinc chromate and lead chromate, respectively. Zinc chromate and lead chromate are the predominate chromium compounds used in pigments but insoluble barium and strontium chromate can also be used. Studies in these workers also found that insoluble chromate exposure increases lung cancer risk and that such risks increase with exposure time[2,3,5,6].

Animal studies support the epidemiologic findings including both the observations that chromate exposure causes lung cancer and that the water insoluble (particulate) compounds are the more potent carcinogenic form. The majority of studies investigating soluble Cr(VI) compounds administered through inhalation, intrabronchial implantation, or intratracheal instillation either showed no effect or small but non significant increases in lung tumour formation[7]. However, numerous studies investigating particulate Cr(VI) compounds such as calcium chromate, strontium chromate, lead chromate and zinc chromate observed significant increases in lung tumours. For example, Levy *et al*[8] showed that intrabronchial implantation of soluble sodium chromate induced only 1 bronchial carcinoma in 100 rats whereas intrabronchial implantation of two different doses of strontium chromate induced 43 and 62 bronchial carcinomas in 99 rats. They reported no increase in bronchial carcinomas after intrabronchial implantation of particulate lead chromate[8], however, three studies injecting lead chromate either subcutaneously or intramuscularly induced a significant increase in sarcomas at the injection site indicating that this compound is also a carcinogen[9-11].

Cell culture data also indicate that the particulate compounds have greater potency. Working with C3H/10T1/2 cells, Patierno *et al*[12] showed that only particulate lead chromate could induce neoplastic transformation while soluble sodium chromate could not. Thus, epidemiology, whole animal and cell culture studies all indicate that the particulate compounds are the most potent carcinogenic forms of Cr(VI) with respect to lung cancer. Interestingly, recent work has shown that soluble Cr(VI) administered chronically in drinking water can induce oral and intestinal tumours[13]. Thus, it appears that while soluble Cr(VI) may only be weakly carcinogenic to the lungs, it may pose a more significant carcinogenic risk if ingested. Studies have not been done of particulate Cr(VI) after oral exposure, but given the low *p*H of the stomach, it is likely that these exposures would ultimately become exposures to soluble Cr(VI) as particulate chromates dissolve at low *p*H. The focus of this review is on chromate-induced lung cancers as the drinking water data are very recent and no studies have been done to clarify the mechanism for these tumours.

Characteristics of chromate-induced lung tumours

Human pathology studies show that Cr(VI) deposits and persists at bronchial bifurcations where Cr(VI)-associated cancers occur[14,15], which is consistent with a particulate chromate exposure. The majority of lung cancers in chromate exposed workers are squamous cell carcinomas, often with multiple tumours[15]. In addition, chromium levels in workers with lung cancer are much higher than in workers without lung cancer[15]. Interestingly, one study found no correlation between chromium accumulation in the lungs of workers and duration of chromium exposure or multiple lung tumours but malignancy stage was significantly associated with chromium accumulation[16]. The association between malignant stage and chromium accumulation was also confirmed in animal studies where a pellet of strontium chromate was inserted into the bronchus of rats[17].

Molecular studies of chromate-induced lung tumours reveal that the tumours exhibit minimal mutations in key oncogenes and tumour suppressor genes but do exhibit genomic instability and epigenetic changes (Table I). For example, there were no significant differences in Bcl-2 and p53 expression between lung tumours from chromate workers, unexposed individuals and individuals with pneumoconiosis[18]. Few *p*53 mutations and no *ras* mutations were observed in tumours from chromate workers[19,20]. By contrast, these tumours exhibited both chromosome instability (CIN) and microsattelite instability (MIN). Hirose *et al*[21] found a statistically significant increase in microsatellite instability. Of 38 chromate-induced lung tumours analyzed, 30 exhibited instability in two or more microsatellite markers (79%) while MIN was observed in only 4 of 26 tumours from non-exposed individuals (15%)[21,22]. MIN also increased with longer exposure time[21] and was strongly correlated with decreased expression of hMLH1[22]. But interestingly, MIN was not significantly associated with hMSH2 repression[22]. In fact, 100 per cent of the tumours without MIN had repressed hMSH2 expression, while only 60 per cent of the tumours with extensive MIN exhibited hMSH2 repression. In

Table I. Characterization of Cr-induced tumours

Study population	Assay	Summary of findings	Ref
19 lung tumours from 18 chromate-exposed workers Unexposed with lung cancer Individuals with pneumoconiosis	Immunohistochemistry for cyclin D1, bcl-2, p53	Cyclin D1 expression significantly increased in chromate workers Bcl-2 and p53 expression not significantly different from controls	18
20 lung tumours from 19 chromate-exposed workers	PCR-SSCP analysis	20% of chromate-induced tumours had *p53* point mutations Fewer *p53* mutations in chromate-induced tumours than in lung tumours without chromate exposure No association between mutations and length of chromate exposure	19
38 lung tumours from 32 workers	PCR-SSCP analysis	No point mutations in *ras* observed in chromate cancers.	20
38 lung tumours from 28 chromate-exposed workers 26 lung tumours from individuals without chromate exposure	Analyzed six microsatellite instability markers	RER statistically higher in chromate vs. non-chromate lung cancer No significant difference in frequency of LOH but LOH frequency still 50-70% Period of chromate exposure in workers with RER were longer than those without RER Workers with longer exposures to Cr(VI) had higher frequency of MIN	21
35 lung tumours from 26 chromate-exposed workers 26 lung tumours from individuals without chromate exposure	Immunohistochemistry for MLH1 and MSH2 COBRA for *MLH1* promotor methylation Microsatellite instability analysis	Chromate-induced tumours showed a statistically significant decrease in expression of hMSH2 and hMLH1 compared to non-chromate lung cancer Only the repression rate for hMLH1 was significantly correlated to MIN 62.5% of the chromate lung cancer exhibited *hMLH1* gene methylation	22
10 lung tumour specimens from chromate workers 90 biopsy specimens from 25 chromate workers	Microscopic X-ray fluorescence analyzer with transmitted X-ray mapping imaging	No correlation between chromium accumulation and chromate exposure, MIN, smoking index or presence of multiple lung carcinoma Amount of chromium accumulation significantly increased according to progression of malignancy	16
38 lung tumours from 31 chromate workers 40 lung tumours from 40 non-exposed individuals	Methylation-specific PCR for *p16* promoter Immunohistochemistry for p16	85.7% of chromate lung cancers had decreased p16 expression Workers with less than 15 years chromate exposure had no *p16* methylation but workers with greater than 15 years exposure had higher levels of *p16* methylation Reduced p16 expression was correlated with methylation at *p16*	23
31 lung tumours from 26 chromate workers 38 adenocarcinomas from non-exposed individuals 46 SCC from non-exposed individuals 89 healthy individuals	PCR genotyping methods for *SP-B* intron-4 polymorphisms	*SP-B* variations were significantly higher in chromate workers with lung cancer compared to unexposed individuals with lung cancer or healthy individuals	24

addition, MIN was not significantly associated with chromium accumulation in the lung[16]. These data suggest there may be a mechanism for MIN that does not involve loss of mismatch repair function. Hirose *et al*[21] also found between 50-75 per cent loss of heterozygosity (LOH) in 6 different loci they examined, however, it was not significantly different compared to tumours without chromate exposure. The lack of statistical significance may suggest that LOH is required for all lung cancers as previous studies have shown that most lung cancers exhibit chromosome instability[25].

Chromate-induced tumours also exhibited changes in gene expression of cyclin D1 and p16[18,23]. Cyclin D1 expression was significantly increased in chromate-induced tumours but was not observed in the normal epithelia adjacent to the tumour[18]. P16 expression was decreased in 86.7 per cent of chromate-induced lung tumours which was associated with promoter methylation[23]. *P16* promoter methylation was linked to exposure time with methylation at the promoter only found in workers exposed to chromate for 15 or more years[23]. Another study investigated variations in the surfactant protein B (*SP-B*) gene. Removal of foreign objects, such as chromate particles, from the upper respiratory tracts requires SP-B and gene variations in *SP-B* could enhance worker's susceptibility to chromate lung cancer[24]. Ewis *et al*[24] found that there was a significant increase in insertion or deletion variations in the *SP-B* gene in workers with chromate-induced lung tumours compared to unexposed individuals and chromate workers without lung cancer[24].

Potential mechanisms of chromate-induced carcinogenesis

The mechanism of chromate-induced carcinogenesis remains unknown. Three well-accepted general paradigms of carcinogenesis include multistage carcinogenesis, genomic instability, and epigenetic modification. Using these three paradigms, we summarize how data collected over the past seven years support or refute these as models for chromate-induced carcinogenesis.

Multi-stage carcinogenesis

Multistage carcinogenesis is a multistep process which involves a series of cellular and molecular changes, as a result of the progressive accumulation of mutations and alterations in protooncogenes and tumour suppressor genes[26]. Thus, for a chemical to have a carcinogenic mechanism that follows this paradigm, it must be capable of inducing a significant number of mutations in target genes.

Only one study investigated Cr(VI)-induced mutation frequency *in vivo* (Table II). This study used the Big Blue transgenic mouse[27]. The authors administered soluble potassium dichromate via intratracheal instillation and found increased mutation frequency in the lung that was dose- and time-dependent[27]. The majority of mutations were G:C targeted base substitutions with a few deletions[27].

Cell culture studies also show that Cr(VI) can induce mutations (Table II). Numerous studies using a shuttle-vector mutagenesis assay found a concentration-dependent increase in mutations after exposure to 10-200 uM soluble Cr(VI)[28-31]. In these studies a SV-40 based plasmid with the *supF* gene was treated extracellularly with Cr(VI) and then transfected into and replicated by a human fibroblast cell line immortalized with SV-40. Mutant clones were then selected for with *Escherichia coli*. These studies found that Cr(VI)-induced mutations were the result of Cr(III)-DNA adducts and were not caused by reactive oxygen species or short-lived Cr(V) and Cr(IV) intermediates[28,31]. They also found that while Cr(III) forms both binary and ternary DNA adducts, it is the ternary DNA adducts that are the most mutagenic and these are generated within 15-60 min after exposure[28,30]. Further, they found that Cys-Cr(III)-DNA adducts and ascorbate-Cr(III)-DNA adducts were 5.3 and 31 times more mutagenic than binary lesions, respectively[28,30]. One study using cysteine as a reducing agent found that single base substitutions of GC to AT or TA were the predominant mutations[28] and another study using ascorbate found a similar level of base substitution and deletion mutations[30].

Three cell culture studies considered mutagenesis in an existing cellular chromosomal locus (Table II). One study investigating both soluble and particulate Cr(VI) compounds found that both potassium chromate (soluble) and barium chromate (particulate) induced mutations in transgenic V79 cells containing the bacterial *gpt* reporter gene. The mutation frequency of potassium chromate peaked at three-fold above background, while the mutation frequency of barium chromate peaked at 3.5-fold[32]. However, the significance of this increased mutation frequency is uncertain because statistical comparisons were apparently not conducted. In contrast to the *in vivo* study, 30-50 per cent of the mutations were deletions[32]. The second study found that the amount of ascorbate present in Chinese hamster ovary cells plays a role in mutation frequency at the *hprt* locus[33]. When cells containing 15 μM ascorbate were exposed to Cr(VI), there was no increase in mutations but if the cells were preloaded with 1.4 mM ascorbate the mutation frequency increased 19.2-fold after exposure to 40 μM Cr(VI). The third study found that exposure to 6 μM sodium chromate for 24 h in Chinese hamster ovary cells induced a 3.5-fold increase in mutation frequency and mutation frequency was attenuated in cells deficient in

Table II. Chromium (VI) - induced mutations

Treatment	Assay (s)	Model system	Summary of effects	Ref
Potassium dichromate 1.7-6.75 mg/kg Intratracheal instillation	LacI gene mutagenesis assay	C57BL/6 Big Blue transgenic mice	Dose and time-dependent increase in mutation frequency in the mouse lung (4.2 fold increase after 4 wk) - 64% G:C to A:T transitions - 26% G:C to T:A transversions - few deletions GSH depletion decreased mutant frequency	27
Potassium chromate 10-100 μM 1 h 2 mM cysteine	Shuttle vector mutagenesis assay	Cr-treated plasmids in HF/SV cells	Concentration-dependent increase in mutations Cr(III)-DNA adducts and not oxidative damage were responsible for mutations Highest mutagenic lesions occurred 15-60 min after exposure Cys-Cr(III)-DNA adducts were more mutagenic than binary Cr(III)-DNA adducts Single base substitutions were most prevalent mutations	28
Potassium chromate 25-200 μM 1 h 2 mM cysteine	Shuttle vector mutagenesis assay	Cr-treated plasmids in HF/SV cells	Cr-DNA adducts induced mutations Adducts arrest replication	29
Sodium chromate 10-200 μM 30 min 1 mM ascorbate	Shuttle vector mutagenesis assay	Cr-treated plasmids in HF/SV cells	Concentration-dependent increase in mutations and replication-blocking lesions Ascorbate-Cr(III)-DNA lesions were more mutagenic and more efficient at inhibiting replication than binary lesions Similar number of base substitutions and deletions	30
Potassium chromate 25-200 μM 1 h 0.2-1 mM ascorbate	Shuttle vector mutagenesis assay	Cr-treated plasmids in HF/SV cells	Concentration-dependent increase in mutations Increased stability of Cr intermediates decreased mutation frequency and did not induce the formation of more potent mutation-inducing lesions	31
Potassium chromate 5-50 μM 2 h Barium chromate 0.05-0.25 μg/cm^2 24 h	Generation of *gpt*-mutants, PCR for deletions	Transgenic, V79 derived, cell line (G12)	3-fold increase in mutation frequency at a dose that induced 40% survival ~50% complete *gpt* deletions 3.5-fold increase in mutation frequency at a dose that induced 75% survival ~30% complete *gpt* deletions	32
Potassium chromate 0-40 μM 1-6 h 15 μM or 1.4 mM ascorbate	*hprt* mutagenesis assay	CHO and V79 cells	No mutations observed in cells not pre-loaded with ascorbate Preloading with ascorbate induced a concentration-dependent increase in mutations at the *hprt* locus	33
Sodium chromate 0-6 μM 24 h	*hprt* mutagenesis assay	CHO cells: AA8 UV-5 (XPD-) UV-41 (XPF-)	Concentration-dependent increase in mutation frequency with 6 μM inducing a 3.5 fold increase in mutation frequency NER-deficient cells showed attenuated mutagenesis	34

nucleotide excision repair (NER)[34]. These data suggest that proficient NER is required for the induction of mutations after Cr(VI) exposure.

Considered together, these studies suggest that Cr(VI) induces mutations, specifically targeting G:C base substitutions. However, the data concerning Cr(VI)-induced deletion mutations are inconsistent. The *in vivo* study using the Big Blue transgenic mouse found few deletion mutations, however, the Big Blue mouse system is insensitive to these types of mutations[27]. The

inconsistency in the *in vitro* data may be due to the different reducing agents used in the experiments. The studies that used cysteine as a reducing agent observed few deletion mutations while the studies that used ascorbate observed comparable numbers of deletion and substitution mutations. In addition, the one cell culture experiment that investigated mutation spectrum found that Cr(VI) induced 30-50 per cent deletion mutations. Thus, Cr(VI) appears to induce deletion mutations except when cysteine is the sole reducing agent.

Interestingly, the data for Cr(VI)-induced tumours appear to contradict the whole animal and cell culture studies. Mutations appear to be infrequent in chromate-induced tumours, though these can be experimentally induced. This discrepancy may indicate that Cr(VI) is only weakly mutagenic, with mutations only occurring at very high doses. Because of the technical challenges of detecting mutations, experimental mutagenesis studies often use very high doses that do not reflect likely exposure scenarios. Indeed for the *in vitro* Cr(VI) studies, the concentrations utilized were often between 25 and 200 μM for short periods of time which is not likely to be an environmentally or occupationally relevant exposure. Exposing cells to this concentration range would result in very little cell survival. For example, exposure to 5 μM sodium chromate for 24 h induces close to 100 per cent cell death in human lung fibroblasts[35,36]. Studies on Cr(VI)-induced toxicity in human lung fibroblasts for shorter periods of time have not been done, but only about 5 per cent of HCT116+ch3 cells (a human colon cancer cell line) survived a 3 h treatment with 20 μM sodium chromate[37]. In addition, the majority of the mutation studies were performed using a shuttle vector system, which does not fully mimic the effects of Cr(VI) in a cell or the ability of DNA repair systems to repair the damage. These differences may contribute to the dramatically higher mutation frequency in the shuttle vector mutagenesis assays compared to the cellular chromosomal locus mutagenesis assay and cause Cr(VI) to appear to be a more potent mutagen in cell culture than it may actually be.

The role of the reducing agent is an interesting factor. The published data suggest that the levels of these reducing agents can affect the mutation spectrum and potency[30,31,33]. However, the full interpretation is complicated by limited data of the levels of these agents in human lung cells. For example, Reynolds *et al*[33] pre-loaded cells with 1.4 mM ascorbate. This level is comparable to ascorbate levels in freshly purified human lymphocytes, however, these ascorbate levels are dramatically higher than those reported in human lung tissue. Specifically, in adults, lung tissue ascorbate levels range from 0.045-0.065 mg/g which is approximately 256 μM ascorbate[38]. Reynolds *et al*[33] found that Cr(VI) increased mutation frequency only occurred in cells preloaded with 1.4 mM ascorbate, a level 5.5-times higher than human lung tissue levels. Cr(VI) was not mutagenic in cells with ascorbate levels lower than 1.4 mM. Thus, the reason mutation levels were low in Cr(VI)-induced human lung tumours may be due to the fact that ascorbate levels in the lung are not high enough for Cr(VI)-induced mutations to occur. Such a conclusion would be consistent with the low mutation rates in cell culture systems without ascorbate. Lymphocytes, although high in ascorbate probably turn over too quickly for mutations to be expressed *in vivo*, which likely explains the absence of significant frequencies of Cr(VI)-induced cancer in these potential target cells.

Interestingly, Reynolds *et al*[33] found that Cr(VI) was not mutagenic in cells without a high dose of ascorbate, while both Klein *et al*[32] and Brooks *et al*[34] reported that Cr(VI) was mutagenic in these cells. More specifically, Klein *et al*[32] found a 3-fold increase in mutation frequency at 40 μM potassium chromate for 2 h while Reynolds *et al*[33] found no increase in mutation frequency with the same treatment for 3 h in cells that were not pre-loaded with ascorbate. The discrepancy could be due to the different cell lines used. Klein *et al*[32] used a CHO cell line that contains the bacterial *gpt* reporter gene, while Reynolds *et al*[33] used the endogenous *hprt* gene in CHO cells to detect mutations. Therefore, the difference in mutation frequency could be due to differences in Cr(VI)-induced mutations in exogenous versus endogenous genes. However, Brooks *et al*[34] used the same CHO cell line as Reynolds *et al*[33] and found a 3.5-fold increase in mutations after a 24 h exposure to Cr(VI) as opposed to 3 h in Reynolds *et al* suggesting that Cr(VI) is mutagenic without excess ascorbate. Given that two studies were positive and the other negative, it seems likely that ascorbate is not required for Cr(VI) mutagenesis, though it may accelerate the process.

If Cr(VI) is indeed a weak mutagen, then the mutation frequency that occurs after occupational Cr(VI) exposure may not be high enough to induce multiple mutations in key genes. For example, based on the chromosomal locus mutagenesis assay in cells that were not loaded with high ascorbate, there was a 2-4 fold increase in mutation frequency above

background[32,34]. Assuming that the spontaneous mutation rate in dividing cells is 1.4 x 10^{-10} mutations per base pair per cell generation and there are ~70,000 genes in the human genome, it is estimated that only one mutation would arise spontaneously in a normal cells lifespan[39]. Therefore, if Cr(VI) increases the normal mutation frequency by 2-4 fold, this would only produce 2-4 mutations in a cell over its lifespan. The likelihood of those 2-4 mutations randomly mutating a key tumour suppressor gene or oncogene seems low. In addition, tumour suppressor genes often require mutations in both alleles in order to be inactivated further decreasing the probability of losing these genes.

On the other hand, it may be that the human tumour studies have not considered enough genes and Cr(VI) simply does not mutate *p53* and *ras*. If this is true there may be a higher mutation rate in other genes. This possibility seems unlikely given that *ras* is mutated in 20-30 per cent of lung cancers[40] and *p53* is mutated in 50-90 per cent of lung cancers[41]. These data would suggest that Cr(VI)-induced tumours are unusual compared to other lung tumours.

The multistage carcinogenesis paradigm requires the stepwise acquisition of mutations in multiple key tumour suppressor and oncogenes. Considering the above studies, it appears that Cr(VI) is not a potent mutagen and is unlikely to induce sufficient mutations for this paradigm to apply. Therefore, we suggest that the multistage carcinogenesis does not fit well for chromate carcinogenesis.

Genomic instability

The genomic instability paradigm argues that disruption of the control of genomic stability results in a cascade of changes in the whole genome[42]. This control can be disrupted by interfering with DNA repair, kinetochore assembly, checkpoints, centrosome duplication, microtubule dynamics and numerous other cellular maintenance processes[42]. In particular, there are two types of genomic instability; microsatellite instability (MIN) and chromosome instability (CIN).

MIN is characterized by changes in the lengths of microsatellites which are series of repetitive non-coding DNA sequences that are abundant in the human genome[43]. DNA polymerases often slip while replicating microsatellites resulting in insertion or deletion loops. In normal cells, these insertion/deletion loops are repaired by mismatch repair (MMR). If cells acquire defective MMR, they are unable to repair these lesions. Unrepaired insertion/deletion loops result in changes in the lengths of microsatellites which is carried through to the whole genome increasing the mutation rate by more than 200-fold[43].

MIN has been observed in Cr(VI)-induced tumours[21]. Seventy nine per cent of the lung tumours from chromate workers exhibited MIN at two or more loci and 18 per cent of the tumours exhibited MIN at all five markers tested[21]. In contrast, only 15 per cent of lung tumours from patients without Cr(VI) exposure exhibited MIN at two or more loci[21]. MIN was associated with length of chromate exposure, with workers with longer exposures exhibiting a higher frequency of MIN[21]. MIN was also associated with hMLH1 repression[22]. No animal studies or cell culture studies have directly investigated the ability of Cr(VI) to induce MIN so it is uncertain if these effects are early or late events in tumourigenesis. This absence of data is largely due to the absence of any sufficient *in vivo* or *in vitro* tests for mismatch repair.

Two cell culture studies investigated the effects of alterations in MMR gene expression. One study reported that MMR-deficient carcinoma cells exhibited decreased cytotoxicity, apoptosis and DNA double strand breaks[37]. In addition, MMR-deficient cells were unable to induce replication arrest due to Cr-DNA adducts[37]. The second study reported that depletion of MLH1 or MSH2 in normal lung cells decreased the formation of CREST(-) micronuclei and DNA double strand breaks after potassium chromate exposure[33]. These data suggest that proficient MMR plays a role in Cr(VI)-induced genotoxicity and cytotoxicity. This observation contrasts with the tumour data because deficient MMR is required for MIN, while proficient MMR is required for double strand break formation which may be a key step in the mechanism of Cr(VI)-induced carcinogenesis.

CIN occurs in the majority of lung cancers and these tumours can have both MIN and CIN[25,44]. CIN includes both numerical and structural changes. Numerical CIN involves the loss and gain of whole chromosomes. Structural CIN involves chromosome translocations and breaks. Exposure to Cr(VI) has been shown to induce both numerical and structural CIN.

Numerical CIN has not been investigated *in vivo* or in chromate-induced tumours. Using cell culture models, five studies assessed numerical CIN after Cr(VI) exposure by counting metaphase chromosomes (Table IIIa). Two studies found that aneuploidy increased in normal human lung fibroblasts after a 30 h

Table IIIa. Genomic instability: Microsatellite instability and numerical chromosome instability

Treatment	Assay(s)	Model system	Summary of effects	Ref
Potassium dichromate 0-30 μM 3 h	Clonogenic survival Western blotting Flow cytometry H2A.X foci formation	A549 human lung carcinoma cells Colon cancer cell lines: HCT116 (MLH1-/-) DLD1 (MSH6-/-) HCT116+ch3 (MLH+) DLD1+ch2 (MSH6+) Mouse embryonic fibroblasts: Mlh-/-, Mlh+/+ Pms-/-, Pms+/+	After Cr(VI) exposure, MMR-deficient cells exhibited: - increased clonogenic survival - decreased apoptosis - inability to block replication as a result of Cr-DNA adducts - decreased DNA double strand breaks	37
Potassium chromate 0.2-5 μM 1-6 h 1.4 mM ascorbate	H2A.X foci formation Micronucleus assay	IMR90 human lung fibroblasts	Depletion of MLH1 or MSH2 decreased micronuclei and double strand break formation	33
Potassium dichromate 0.25-1 μM 30 h	Chromosome counting	MRC-5 human lung fibroblasts	Increase in aneuploid cells Increase in hypodiploid cells and no increase in hyperdiploid cells	45
Potassium dichromate 0.25-1 μM 30 h	Chromosome counting Anaphase-telophase assay	MRC-5 human lung fibroblasts	Increase in aneuploid cells, specifically hypodiploid cells Increase in chromatin bridges, lagging chromosomes and lagging fragments	46
Lead chromate 0.1-1 μg/cm^2 24-120 h	Chromosome counting Clonogenic aneuploidy Mitotic stage analysis Centrosome analysis	WTHBF-6 human lung fibroblasts	Concentration- and time-dependent increase in: - aneuploid cells including hypodiploid and polyploid cells - centrosome number in both interphase and mitotic cells - abnormal mitotic figures including disorganized anaphase and mitotic catastrophe Aneuploid cells were able to survive and form colonies	17
Lead chromate 0.1-1 μg/cm^2 24-120 h	Chromosome counting Chromosome damage Clonogenic aneuploidy Mitotic stage analysis Western blot for Mad2 expression	WTHBF-6 human lung fibroblasts	Concentration- and time-dependent increase in premature centromere division, centromere spreading and premature anaphase Lead chromate disrupts mitotic progression with an increase of cells in anaphase Decreased Mad2 expression Time- and concentration-dependent increase in tetraploid cells and tetraploid cells persist and form colonies SAC bypass due to chromium and not lead ions or the particle	48
Lead chromate 1-10 μg/cm^2 120 h	Transformation assay Chromosome damage Centrosome analysis	BEP2D human lung epithelial cells	Chronic exposure to lead chromate induced loss of contact inhibition and anchorage-independent growth Foci cells exhibited aneuploidy and centrosome amplification	49

exposure to soluble chromate with a specific increase in hypodiploid cells but no increase in hyperdiploid cells[45,46]. Two studies found that chronic exposure to particulate chromate induced concentration- and time-dependent increases in aneuploidy in normal human lung fibroblasts. However, in contrast to the soluble chromate study, these studies reported increases in both hypodiploid and tetraploid cells[47,48]. These particulate

chromate-induced aneuploid cells were able to survive and form colonies[47,48]. The fifth study showed that human lung epithelial cells transformed with lead chromate also had increased aneuploidy further indicating that the aneuploid phenotype persists[49].

There are a number of mechanisms that can give rise to numerical CIN, including centrosome amplification, spindle assembly checkpoint bypass, malfunctions in sister chromatid cohesion and abnormalities in kinetochore structure or function[42]. Centrosome amplification can induce aneuploidy through multipolar spindle formation and division[50]. Two studies considered centrosome amplification after Cr(VI) exposure[47,49]. Holmes *et al*[47] reported a concentration- and time-dependent increase in centrosome amplification in human lung fibroblasts exposed to particulate chromate. Centrosome amplification was also observed in human lung epithelial cells transformed by particulate chromate indicating that centrosome amplification may be an early event in Cr(VI)-induced carcinogenesis and that this phenotype persists[49]. One study considered spindle assembly checkpoint bypass as a potential mechanism of Cr(VI)-induced aneuploidy[48]. This study reported spindle assembly checkpoint bypass after chronic particulate chromate exposure with concentration- and time-dependent increases in premature anaphase, premature centromere division, centromere spreading and the total number of cells in anaphase. In addition, expression levels of Mad2, an important protein involved in the regulation of the spindle assembly checkpoint, were suppressed confirming spindle assembly checkpoint bypass on a molecular level[48]. This study also showed that Cr(VI) and not the lead cation or internalized particles were responsible for the spindle assembly checkpoint bypass[48]. The observed increase in centromere spreading may also indicate that chromium induces malfunction in sister chromatid cohesion. Consistent with spindle assembly checkpoint bypass and chromosome segregation malfunctions, Seoane *et al*.[46] showed increased chromatin bridges, lagging chromosomes and lagging fragments after a 30 h soluble chromate exposure in human lung fibroblasts.

The induction of translocations is the gold standard for evaluating and measuring structural CIN but so far no studies have investigated the ability of Cr(VI) to cause translocations. The majority of work investigating structural chromosomal effects has considered the induction of chromosomal aberrations which can lead to structural CIN (Table IIIb). Two different assays have been used to assess chromosome damage: The chromosome damage assay and the micronucleus assay. The chromosome damage assay is a more sensitive assay and provides information on the kind of damage formed. The micronucleus assay is less sensitive but is quicker. A wealth of data using the chromosome damage assay shows that both particulate and soluble Cr(VI) induce chromosomal aberrations in human lung fibroblasts and human lung epithelial cells[35,51-58]. Furthermore, these studies show that the lead cation and internalized particles do not contribute to Cr(VI)-induced clastogenicity and that all of the effects are due to partial dissolution of chromate particles releasing extracellular Cr(VI).

Three studies have investigated chromosome damage *in vivo* using the micronucleus assay. The National Toxicology Program (NTP) report, 2007 showed that soluble chromate administered orally through drinking water induced a concentration-dependent increase in micronuclei in normochromatic erythrocytes in *am*3-C57BL/6 male mice but only a small increase in micronuclei in $B6C3F_1$ mice and no increase in BALB/c mice[59]. A second study in the NTP report showed no increase in micronuclei in normochromatic erythrocytes after exposure to soluble chromate for 3 months through drinking water in F344/N rats and $B6C3F_1$ mice[59]. The third study showed that exposure to a single dose of soluble chromate delivered by intraperitoneal injection increased micronuclei frequency in polychromatic erythrocytes in BDF_1 mice[60].

The results from these *in vivo* studies are inconsistent and have some shortcomings in regard to Cr(VI)-induced lung cancer, especially since the NTP report was focused on the effects of Cr(VI) in drinking water. First, these studies used soluble chromates and administration routes that do not directly expose the lung. In addition, the authors investigated micronuclei formation in erythrocytes which are not the target cells in chromate-induced lung tumours. One cell culture based study using the micronucleus assay is consistent with the chromosome damage studies confirming that Cr(VI) induces chromosome aberrations[33]. Even though the *in vivo* data are inconsistent, the cell culture data clearly show that particulate and soluble chromate induce chromosome damage in human lung cells.

Cr(VI)-induced chromosome aberrations are most likely caused by Cr(VI)-induced DNA double strand breaks (Table IIIc). No *in vivo* studies have specifically investigated double strand break formation. Several recent cell culture studies demonstrated that both

Table III b. Genomic instability: Structural chromosome instability manifested as chromosome damage

Treatment	Assay(s)	Model system	Summary of effects	Ref
Lead chromate 0.1-5 μg/cm² 24 h Sodium chromate 1-10 μM 24 h	Chromosome damage	Primary human lung cells	Lead chromate and sodium chromate induced chromosome damage in a concentration-dependent manner	35
Lead chromate 0.1-5 μg/cm² 24 h Sodium chromate 1-10 μM 24 h	Chromosome damage	Primary human lung cells and WTHBF-6 human lung fibroblasts	Chromosome damage levels were similar in primary human lung cells and WTHBF-6 (hTERT+) cells WTHBF-6 cells are as a useful lung cell model	51
Lead chromate 0.1-5 μg/cm² 24 h Sodium chromate 1-5 μM 24 h	Chromosome damage	WTHBF-6 human lung fibroblasts	Cr(VI) is the proximate clastogen Lead ions are not involved in the clastogenicity of lead chromate	52
Barium chromate 0.1-5 μg/cm² 24 h	Chromosome damage	WTHBF-6 human lung fibroblasts	Barium chromate induced concentration-dependent increases in chromosome damage	53
Lead chromate 0.1-5 μg/cm² 24 h Barium chromate 0.1-5 μg/cm² 24 h	Chromosome damage	WTHBF-6 human lung fibroblasts	Barium chromate was more genotoxic than lead chromate	54
Lead chromate 0.1-5 μg/cm² 24 h Sodium chromate 1-2.5 μM 24 h	Chromosome damage Chromium particle uptake	WTHBF-6 cells	Chromosome damage was due to external dissolution of lead chromate particles Particle-cell interaction was not required for chromosome damage induction	55
Lead chromate 0.1-5 μg/cm² 24 h	Chromosome damage Neutral comet assay H2A.X foci formation	WTHBF-6 human lung fibroblasts	Concentration-dependent increase in chromosome damage Concentration-dependent increase in DNA double strand breaks ATM is phosphorylated in response to DSB	56
Lead chromate 0.1-1 μg/cm² 24-72 h Sodium chromate 0.5-2.5 μM 24-72 h	Chromosome damage	WTHBF-6 human lung fibroblasts	Lead chromate induced a concentration-dependent increase in chromosome damage which persisted over time Sodium chromate-induced chromosome damage decreased over time	57
Lead chromate 0.5-50 μg/cm² 24 h Sodium chromate 0.5-10 μM 24 h	Chromosome damage	BEP2D lung epithelial cells	Lead chromate and sodium chromate induced similar amounts and spectrum of chromosome damage in human lung epithelial cells	58
Sodium dichromate 1.7-20.9 mg/kg in rats 3.1-27.9 mg/kg in mice 3 months orally	Micronucleus assay	F344/N rats and $B6C3F_1$ mice	No increase in micronuclei in normochromatic erythrocytes	59
Sodium dichromate 2.8-8.7 mg/kg 3 months orally	Micronucleus assay	$B6C3F_1$, BALB/c, and *am3*-C57BL/6 mice	Concentration-dependent increase in micronuclei in normochromatic erythrocytes in *am3*-C57BL/6 male mice	59

Contd....

Treatment	Assay(s)	Model system	Summary of effects	Ref
			Small (NS) increase in micronuclei in $B6C3F_1$ mice and no increase in BALB/c mice	
Potassium dichromate 50 mg/kg i.p. injection 1 dose	Micronucleus assay	40 BDF1 mice	Increased frequency of micronuclei in polychromatic erythrocytes	60
Potassium chromate 0.2-5 μM 1-6 h 1.4 mM ascorbate	Micronucleus assay	IMR90 human lung fibroblasts	Preloading with ascorbate increased CREST-negative micronuclei formation Depletion of MLH1 or MSH2 decreased micronuclei	33

soluble and particulate Cr(VI) induce DNA double strand breaks measured by gamma-H2A.-X foci formation or with the single cell gel electrophoresis assay (comet assay)[33,37,56,61-64]. These studies show that the breaks only form during S and G2 phases as a result of either excision repair of crosslinks/ternary adducts or collapsed replication forks due to repeated cycles of futile MMR attempting to repair these lesions[33,34,63]. In addition, failure of homologous recombination repair of DNA double strand breaks leads to more complex and increased chromosome damage and neoplastic transformation[61,65]. By contrast, failed NHEJ repair has no effect on chromosomal aberrations[66].

The Cr(VI)-induced tumour and cell culture studies show that Cr(VI) induces genomic instability but further work needs to be performed to elucidate the specific mechanism. Studies show that the Cr(VI)-induced tumours exhibit MIN implying MMR inactivation, however, further work needs to be performed on the mechanism of MMR inactivation. Current data suggest that proficient MMR is required to produce double strand breaks and no studies have demonstrated how or why Cr(VI) would target MMR genes. Cell culture studies show that Cr(VI) induces aneuploidy, centrosome amplification and spindle assembly checkpoint bypass but these phenotypic changes still need to be confirmed in the tumours. In addition, data suggest that Cr(VI) induces structural chromosome instability because it induces chromosome damage and DNA double strand breaks but this needs to be confirmed by investigating the ability of Cr(VI) to induce translocations. However, considering all of the data, the genomic instability paradigm is a possible model for Cr(VI)-induced carcinogenesis.

Epigenetic modifications

Epigenetic modifications in cancer are changes in cellular functions that occur without altering the genetic material resulting in tumourigenesis[67]. Examples of epigenetic changes that could drive tumour formation include altered DNA methylation or acetylation, protein phosphorylation status changes, growth stimulation, and altered gene expression or signaling pathways. Epigenetic changes, including growth stimulation and enhanced survival, changes in phosphorylation and methylation status and altered gene expression and signaling pathways, have been considered after Cr(VI) exposure.

Five recent studies have considered the potential role of growth stimulation, escape from growth arrest and apoptosis inhibition after Cr(VI) exposure. All were cell culture studies (Table IVa). Two studies investigated the hypothesis that the cation released from the partial dissolution of particulate Cr(VI) compounds caused Cr(VI) damaged cells to survive. These studies used lead chromate as a model compound in human lung fibroblasts and found that lead did not promote survival of Cr(VI)-damaged cells or stimulate the growth of Cr(VI)-damaged cells[36,68]. Three studies investigated the hypothesis that Cr(VI) could inhibit apoptosis. One study looked at direct inhibition by soluble Cr(VI) in human lung epithelial cells[69]. It reported that soluble Cr(VI) induced NF-kB activation which inhibited apoptosis possibly through the inhibition of p53 activation[69]. Two studies considered effects on apoptosis in cells that survived Cr(VI) treatments[70,71]. One study found that human skin fibroblasts which survived a highly toxic dose of sodium chromate were resistant to apoptosis and had at least a 2-fold change in gene expression in genes involved in DNA repair, apoptosis and cell cycle regulation[70]. Son *et al*[71] generated Cr(VI)-resistant clones from human kidney cells after three consecutive chromium trioxide treatments. These clones were resistant to the cytotoxicity of chromium trioxide and grew at a similar rate in media containing high concentrations of chromium oxide as cells in treatment-free media[71].

Two studies investigated the potential effects of Cr(VI) on phosphorylation (Table IVb). One was in cell culture and the other was *in vitro*. The cell culture study showed that tyrosine phosphorylation increased in a

Table IIIc. Genomic instability: Structural instability manifested as DNA double strand breaks

Treatment	Assay(s)	Model system	Summary of effects	Ref
Potassium chromate 0.2-5 μM 1-6 h 1.4 mM ascorbate	H2A.X foci formation	IMR90 human lung fibroblasts	Preloading with ascorbate increased H2A.X foci formation Depletion of MLH1 or MSH2 decreased double strand break formation Double strand break formation occurred in S phase	33
Potassium dichromate 0-30 μM 3 h	H2A.X foci formation	A549 lung carcinoma cells Colon cell lines: HCT116 (*MLH1-/-*) DLD1 (*MSH6-/-*) HCT116+ch3 (*MLH+*) DLD1+ch2 (*MSH6+*) Mouse embryonic fibroblasts: *Mlh-/-, Mlh+/+ Pms-/-, Pms+/+*	Cr(VI) induced DNA double strand breaks Cells deficient in MMR exhibited decreased DNA double strand breaks	37
Lead chromate 0.1-5 μg/cm^2 24 h	Neutral comet assay H2A.X foci formation	WTHBF-6 human lung fibroblasts	Concentration-dependent increase in DNA double strand breaks ATM is phosphorylated in response to double strand breaks	55
Lead chromate 0.1- 1 μg/cm^2 120 h	Transformation assay	WTHBF-6 human lung fibroblasts ATLD2 (*Mre11-*) human skin fibroblasts BJhTERT human skin fibroblasts	Lead chromate induced loss of cell contact inhibition and anchorage independence in Mre11-deficient cells	61
Sodium chromate 0-6 μM 1-24 h	Western blot *In vitro* kinase assay PS translocation assay Cell growth analysis Cell cycle analysis	Normal human skin fibroblasts ATM null human skin fibroblasts	ATM is activated after Cr exposure and phosphorylates p53 at Sre-15 and Chk2 at Thr-68. ATM-deficient cells were resistant to Cr apoptosis but exhibited a prolonged growth arrest	62
Sodium chromate 3-6 μM 1 or 3 h	Neutral comet assay H2A.X foci formation Cell cycle analysis	Normal human skin fibroblasts, *ATM-/-* human skin fibroblasts	Cr(VI) induced DNA double strand breaks Cr(VI)-induced DNA double strand breaks are S phase-dependent	63
Potassium chromate 10-40 μM 30 min - 24 h	Comet assay H2A.X foci formation Flow cytometry S-phase arrest	SV40-immortalized human skin fibroblasts from A-T heterozygote and from A-T patient HeLa cells	Cr(VI) induced DNA double strand breaks ATM is activated after exposure to Cr and is required for S-phase checkpoint activation	64
Lead chromate 0.1-1 μg/cm^2 24 h	Chromosome damage	CH ovary cells: AA8 (WT) irs1SF (*XRCC3-*)1SFwt8 (*XRCC3+*) CH lung cells: V79 (WT) irs3(*RAD51C-*) irs3#6 (*RAD51C+*)	Cells deficient in RAD51C or XRCC3 exhibit increased chromosome damage and chromatid exchanges after treatment with lead chromate	65
Lead chromate 0.1-10 μg/cm^2 24 h	Chromosome damage	CH ovary cell: CHO-K1 (WT), xrs6 (*Ku80-*), 2E (*Ku80+*)	Ku80-deficient cells exhibit similar levels of chromosome damage compared to wild-type	66

time-dependent manner in human lung tumour cells after exposure to 300 μM Cr(VI) for 5-60 min[72]. More prolonged exposures reduced tyrosine phosphorylation levels back to basal levels[72]. The data suggest that the increased phosphorylation was due to the production of hydrogen peroxide and the hydroxyl radical during the reduction of Cr(VI)[72]. The *in vitro* study also showed that Cr(VI) and Cr(V) induced phosphorylation by facilitating the transfer of a phosphate to the hydroxyl group of serine, threonine and tyrosine by alkali hydrolysis[73]. However, in contrast to the cell culture study, this *in vitro* study showed that Cr(VI) and more

efficiently Cr(V) induced the non-enzymatic phosphorylation of bovine serum albumin (BSA)[73].

Studies performed in Cr(VI)-induced tumours showed that methylation of the p16 promoter increased with greater than 15 years of exposure to chromate[23] suggesting that methylation may be an important mechanism for Cr(VI)-induced lung cancer. Cr(VI)-induced methylation has not been considered in experimental animals. These effects have not been considered *in vivo*. Only one cell culture study has investigated the potential effects of Cr(VI) on methylation (Table IVb). It considered Cr(VI) effects on methylation in a transgenic Chinese hamster lung cell line with a bacterial *gpt* reporter gene. The study investigated both particulate and soluble chromate and reported partial methylation in the *gpt* gene after soluble Cr(VI) exposure, but no methylation changes after particulate Cr(VI) exposure[32]. These observations may reflect real differences between the two types of chromate or alternatively may be related to exposure time as soluble chromate treatment was only for 2 h while the barium chromate treatment was for 24 h[32].

Three studies have utilized microarrays to investigate gene expression changes after Cr(VI) exposure (Table IVc). One study considered expression in an *in vivo* model and evaluated the expression of 56 genes in lung and liver tissue after 3 days of intratracheal instillation of soluble chromate in male Sprague-Dawley rats[74]. Genes induced in the lung that are involved in Cr(VI) reduction, stress response, apoptosis, cell cycle control and DNA repair were altered. No changes were observed in the liver. Two studies considered gene expression changes after soluble chromate exposure in cultured cells. Ye and Shi[75] found changes in genes involved in carcinogenesis such as Src, MAPK and its related proteins, cell cycle regulation, checkpoint suppressor 1, wnt-13 and carcinoma-associated antigen GA733-2 after a 2 h exposure to 300 μM soluble chromate in human lung tumour cells. Andrew *et al*[76] considered 1200 genes and found expression changes in 44 genes after exposing immortalized human lung epithelial cells to 10 μM Cr(VI).

Table IVa. Epigenetic changes: Escape from growth arrest/apoptosis or growth stimulation

Treatment	Assay(s)	Model system	Summary of effects	Ref
Lead chromate 0.1-5 μg/cm² 24 h Sodium chromate 1-10 μM 24 h	Clonogenic survival	WTHBF-6 human lung fibroblasts	Lead ions do not promote Cr(VI)-damaged cells to survive Cr(VI) ions are responsible for lead chromate-induced cytotoxicity	36
Lead chromate 0.1-5 μg/cm² 24 h Sodium chromate 1-10 μM 24 h	Clonogenic survival Clastogenicity Growth curves Cell cycle analysis Mitotic index	WTHBF-6 human lung fibroblasts	Lead ions do not stimulate Cr(VI)-damaged cells to grow Cr(VI) ions cause growth inhibition and arrest	68
Sodium dichromate 1-20 μM 36 h	Morphological changes indicative of apoptosis PARP cleavage TUNEL analysis	BEAS-2B lung epithelial cells: IKK = normal NF-kB activity; KM = low NF-kB activity	Cr(VI) induced NF-kB activation NF-kB activation prevented cells from undergoing apoptosis NF-kB deficient cells activated p53 whiles NF-kB proficient cells did not	69
Sodium chromate 1-9 μM 4-24 h	Phosphatidylserine translocation assay Clonogenic survival Growth curves Microarray	BJ-hTERT derived B-5Cr (cells that survived 99% clonogenic lethality)	B-5 Cr cells were resistant to apoptosis and exhibited increased clonogenic survival 2 fold difference in regulation of genes involved in DNA repair, apoptosis and cell cycle regulation	70
Chromium trioxide 5-100 μM 1-7+ wk	Generation of resistant clones Clonogenic survival Growth curve Toxicity analysis curve	293 human kidney cells	Generated Cr(VI)-resistant clones after consecutive treatments with chromium trioxide Cr(VI)-resistant cells grew at the same rate regardless of whether chromium trioxide was present or absent in the media Resistance was specific to Cr(VI)	71

Table IVb. Epigenetic changes: Phosphorylation and methylation changes

Treatment	Assay(s)	Model system	Summary of effects	Ref
Potassium dichromate 300 μM 5-60 min	Western blot for phosphotyrosine expression	A549 human lung carcinoma cells	Time-dependent increase in tyrosine phosphorylation after Cr(VI) exposure Tyrosine phosphorylation is the result of H_2O_2 and OH radical production Tyrosine phosphorylation returned to basal level after prolonged exposure	72
Potassium dichromate Cr(V) species 100-500 μM 2 min - 18 h	*In vitro* BSA phosphorylation	Bovine serum albumin + radiolabeled ATP	Concentration-dependent non-enzymatic phosphorylation of BSA by Cr(VI) and Cr(V) Cr(V) compounds were more efficient at phosphorylating BSA compared to Cr(VI) Phosphate transferred by alkali hydrolysis to hydroxyl groups of serine/ threonine and tyrosine	73
Potassium chromate 5-50 μM 2 h Barium chromate 0.05-0.25 μg/cm^2 24 h	Southern blot for the detection of methylation variants	Transgenic, V79 derived, cell line (G12)	Exposure to potassium chromate for 2 h induced partial methylation at gpt locus Exposure to barium chromate for 24 h induced no methylation changes	32

Table IVc. Epigenetic changes: Gene expression changes

Treatment	Assay(s)	Model system	Summary of effects	Ref
Sodium dichromate 1.25-2.5 mg/kg intratracheal instillation 3 days	Microarray	Sprague-Dawley rats	No change in gene expression in the liver Expression of 56 genes increased in lung after Cr exposure Genes involved in Cr(VI) metabolism, stress response, protein and DNA repair, signal transduction, apoptosis and cell cycle regulation were altered	74
Potassium dichromate 300 μM 2 h	Microarray	A549 human lung carcinoma cells	Activation of genes involved in tumourigenesis included NEN-1, cystatin M, carcinoma-associated antigen GA733-2, breast tumour anti-antigen, checkpoint suppressor 1, wnt-13 and cytochrome c-like polypeptide	75
Sodium dichromate 10 μM 4 h	1200 gene nylon array	BEAS-2B human lung epithelial cells	Chromium altered the expression of 44 genes Changes in mRNA expression induced changes at the protein level	76

Alteration of cell signaling pathways is another epigenetic mechanism. Three major MAPK pathways are the ERK, JNK and p38 pathway. These pathways elicit a variety of responses depending on the activating signals but they range from cell growth and proliferation to apoptosis and differentiation. Many cell culture studies have investigated the effects of Cr(VI) on the MAPK pathways (Table IVd). These effects have not been considered *in vivo*. These studies found that JNK, p38 and to a lesser degree ERK was activated in a concentration and time-dependent manner by Cr(VI)[77-80]. p38 and JNK were activated in response to oxidative stress while ERK activation was not affected by oxidative stress[77]. Activation of JNK, p38 and ERK were not responsible for Cr(VI)-induced cytotoxicity and therefore may promote cell survival and subsequently carcinogenesis[77,78]. Another study investigating the effects of lower levels of Cr(VI) on the JNK pathway reveal that Cr(VI) and not reactive oxygen species specifically activated JNK through the Lck/Fyn-Cas-Crk signaling cascade; however, there was no connection between JNK activation and carcinogenesis[81]. This study also showed that in addition to activating JNK, Lck can also activate STAT3 which induces the transactivation of IL-6. Activation of IL-6 for prolonged periods of time may induce chronic

Table IVd. Epigenetic changes: MAPK and NFkB signaling

Treatment	Assay(s)	Model system	Summary of effects	Ref
Potassium dichromate 10-80 μM 1-12 h	Clonogenic survival Kinase activity assay	CL3 non-small cell lung carcinoma cells	Concentration- and time-dependent increases in JNK, p38 and only small in creases in ERK p38 and JNK were induced in response to oxidative stress while ERK was unaffected by oxidative stress Activation of JNK, p38 or ERK were not responsible for Cr(VI)-induced cytotoxicity	77
Potassium dichromate 10-80 μM 1-12 h	MTT assay Clonogenic survival Annexin V apoptosis assay Kinase activity assay	CL3 non-small cell lung carcinoma cells	Cr (VI)-activated JNK was not involved in apoptosis ERK, JNK and p38 were not involved in Cr (VI)-induced cytotoxicity	78
Sodium dichromate 10-80 μM 1-12 h	Western blot Co-immunoprecipitation	A549 human lung carcinoma cells	Concentration- and time-dependent increase in the activation of JNK and c-Jun No increase in p38 activation ASK1 was activated by dissociation from its regulatory partner and was responsible for JNK activation	79
Potassium dichromate 0.2-200 μM 1 h	Western blot	Normal human small airway epithelial cells	Increased activation of p38, JNK and ERK	80
Potassium dichromate 10 μM 5-120 min	Kinase activity assays	A549 human lung carcinoma cells	Nontoxic doses of Cr(VI) induced both JNK and reactive oxygen species Cr(VI) induced Fyn and Lck (but not Src and Cas) and were required for JNK activation Effects are specific to Cr(VI) and not due to ROS	81
Potassium dichromate 10 μM 1-72 h	Western blot Immunofluorescence	BEAS-2B human lung epithelial cells and primary HBE cells	Cr(VI) activated Lck inducing prolonged activation of STAT3 and transactivation of IL-6 Jak was not activated	82
Soluble chromate 0.02-5 μg/ml 1 min -12 h	Mobility shift assay Reporter gene activity assay	RAW264.7 mouse macrophages and mouse JB6 skin fibroblasts	NF-kB and AP-1 activation was time- and concentration-dependent OH radical scavengers inhibited Cr(VI) activation of NF-kB and AP-1 Inhibitors of p38 but not Erk reduced AP-1 activation	83
Potassium dichromate 0.1-200 μM 3-16 h	Electrophoretic mobility shift assay Western blot DNA fragmentation assay Non-radioactive ELISA	BEAS-2B human lung epithelial cells and A549 human lung carcinoma cells	Concentration-dependent activation of NF-kB and p53 Concentration-dependent decrease in cell proliferation and increase in apoptosis	84
Potassium dichromate 12.5-800 μM 6 h	Electrophoretic mobility shift assay	A549 human lung carcinoma cells	DNA binding of NF-kB increased in a concentration- dependent manner Reactive oxygen scavengers inhibited NF-kB activation	85
Lead chromate 10-50 μg/ml 0.5-12 h	Luciferase assay for NF-kB and AP-1	RAW264.7 mouse macrophages	NF-kB and AP-1 were activated by lead chromate	86

inflammation and assist in the progression of lung cancer[82]. Hodges *et al*[79] showed that JNK and c-Jun were activated in a time-dependent manner after exposure to sodium dichromate however, there was no effect on p38. The authors also showed that ASK1 was an upstream activator of JNK1[79]. Studies suggest that NF-kB was also activated by both soluble and particulate Cr(VI) and it was activated in response to ROS via the p38 pathway[83-86]. However, the activation of NF-kB may not play a role in tumourigenesis because activation of NF-kB was associated with a decrease in cell proliferation and an increase in apoptosis[84].

Altogether these data show that chromate has the ability to induce epigenetic changes. However, at this time the consequences of these effects and their impacts on Cr(VI)-carcinogenesis remain unknown. For example, the effects on methylation may be meaningful if they were to silence a gene like p16 or MLH1 for lengthy periods of time while Cr causes DNA damage and chromosome instability. On the other hand, the effect on methylation may be merely transient, as suggested by the soluble and particulate data, and only occur briefly after the first couple hours of exposure and have no long term effects. It is also possible that some of these changes may inhibit tumour progression rather than promote it. For example, the activation of NF-kB may induce apoptosis rather than cell survival. More research needs to be performed in order to better elucidate the roles of epigenetic modification in chromate-induced carcinogenesis. Thus, epigenetic changes may have some importance as a factor in Cr(VI) carcinogenesis, but given the potency of Cr(VI) as a clastogenic agent and the uncertain impact of the epigenetic changes, it does not appear to be sufficient to explain Cr(VI) carcinogenesis on its own.

Conclusion

Based on the recent chromate literature discussed above, we suggest that the paradigm that best describes chromate-induced lung cancer is genomic instability. In the Fig., we propose a potential mechanism of particulate chromate-induced carcinogenesis. Specifically, particulate Cr(VI) (1) dissolves outside the cell into the chromate anion (2A) and cation (2C). The cation enters into the cell via a channel protein (3-4) and intact particles enter into the cell via phagocytosis (2B) but both appear to have no effect[36,52,55]. The chromate anion, on the other hand, is the proximate genotoxic agent and enters into the cell

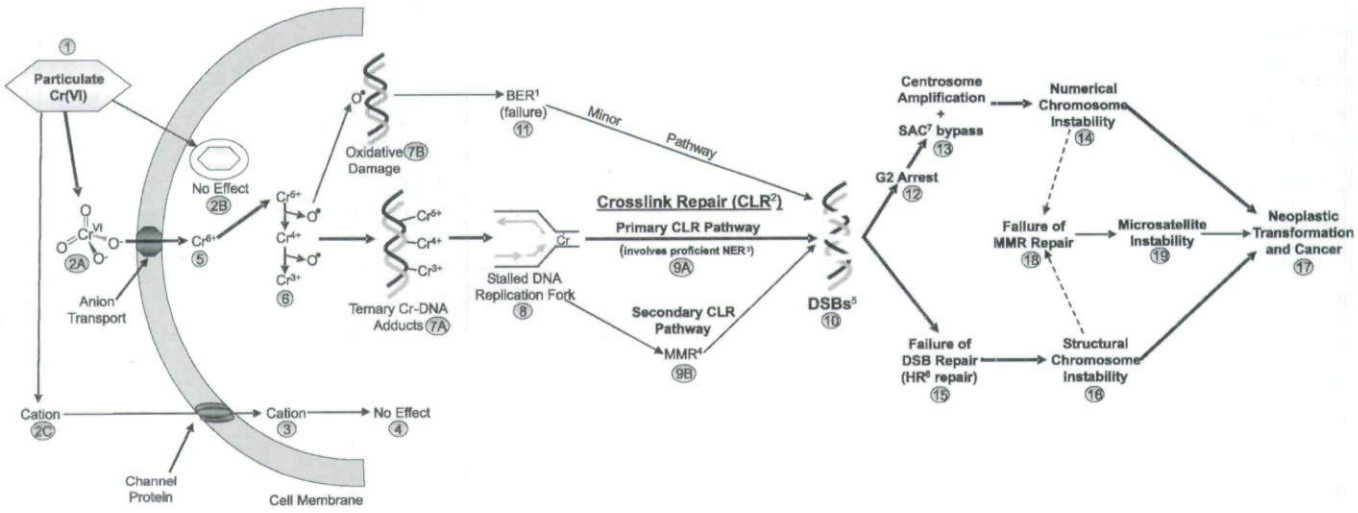

Fig. Proposed mechanism of Cr(VI)-induced carcinogenesis. Particulate Cr(VI) (1) partially dissolves outside the cell producing a chromate anion (2A) and a cation (2C). The cation enters into the cell through a channel protein (3,4) and intact particles are phagocytosed into the cell (2B). Both appear to have no adverse effect on the cell. The chromate anion enters the cell through an anion transporter (5) and is rapidly reduced to Cr(III) generating Cr(V), Cr(IV) and reactive oxygen species in the process (6). Cr(III) and possibly Cr(V) and Cr(IV) form ternary Cr-DNA adducts (7A) leading to a stalled DNA replication fork (8). These ternary adducts can be repaired by crosslink repair involving nucleotide excision repair (9A) or mismatch repair (9B) or possibly both. Both pathways cause a DNA double strand break during the repair process (10). The failure of base excision repair to repair oxidative damage could also contribute to DNA double strand break formation, but this is likely to be a minor component as it requires failure of repair (7B, 11). These DNA double strand breaks induce a prolonged G2 arrest (12) leading to both centrosome amplification and spindle assembly checkpoint bypass (13). Centrosome amplification and spindle assembly checkpoint bypass both lead to numerical chromosome instability (14) and ultimately neoplastic transformation and cancer (17). The failure to properly repair the DNA double strand breaks (15) results in structural chromosome instability (16) which also contributes to neoplastic transformation and cancer (17). Lastly, we propose that failure of mismatch repair (18) is the result of chromosome instability and mismatch repair failure leads to microsatellite instability (19) which may also contribute to neoplastic transformation and cancer (17). ([1]BER = Base excision repair; [2]CLR = Crosslink repair; [3]NER = Nucleotide excision repair; [4]MMR = Mismatch repair; [5]DSBs = Double strand breaks; [6]HR = Homologous recombination; [7]SAC = Spindle assembly checkpoint).

via an anion transporter (5)[52]. Once inside the cell, Cr(VI) is rapidly reduced to Cr(III), forming Cr(V), Cr(IV) and reactive oxygen species in the process (6)[87]. Cr(III), as well as Cr(V) and Cr(IV), can form ternary Cr-DNA adducts (7A) which lead to stalled DNA replication forks (8)[28-31]. The Cr-DNA adducts can be repaired through crosslink repair which involves proficient nucleotide excision repair and results in a DNA double strand break (9A-10)[34]. Mismatch repair also tries to repair Cr-DNA adducts but fails to repair them and undergoes a series of futile repair cycles that ultimately fail and collapse the replication fork also leading to a DNA double strand break (9B-10)[33]. A third pathway in the formation of double strand breaks could be the result of failure of base excision repair to properly repair oxidative damage (7B, 11, 10). Next, we propose that the DNA double strand breaks lead to a G2 arrest (12). With chronic exposure, this G2 arrest becomes prolonged and induces both centrosome amplification and spindle assembly checkpoint bypass (13)[47,48]. Centrosome amplification and spindle assembly checkpoint bypass induce numerical chromosome instability ultimately leading to neoplastic transformation and cancer (14,17)[47-49]. In addition, misrepair of double strand breaks can cause structural chromosome instability also contributing to neoplastic transformation and cancer (15-17)[49,61].

Interestingly, chromate-induced tumours exhibit MIN as well as CIN[21], however, the formation of Cr(VI)-induced DNA double strand breaks requires proficient, functional MMR[33]. Therefore, we propose that failure of MMR leading to MIN is a later change in the cells and probably a consequence of chromosome instability (18-19).

Epigenetic changes and mutagenesis may contribute to this carcinogenic mechanism. For example, the activation of MAPK pathways may promote the survival of cells with Cr(VI)-induced CIN or DNA methylation may alter expression of genes and promote the growth of cells with Cr(VI)-induced CIN into a tumour. Mutagenesis may have some contribution if exposures are high enough. A mutation in a gene involved in MMR in the later stages of tumour progression could result in the MIN phenotype observed in the tumours. DNA methylation of MMR genes could also contribute to the later acquisition of the MIN phenotype. More work is needed to understand how the epigenetic changes are playing a role and if significant mutagenesis occurs in relevant genes at likely exposure levels.

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