Neoplastic Transformation of Human Bronchial Cells by Lead Chromate Particles

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Particulate hexavalent chromium (Cr(VI)) is a well-established human lung carcinogen with widespread exposure among people in occupational settings and the general public. However, no studies have examined the chromate-induced malignant transformation of human lung epithelial cells, its predominant target. Human papillomavirus-immortalized human bronchial epithelial (BEP2D) cells were used to better understand the mechanisms involved in human bronchial carcinogenesis induced by particulate chromate. We found that aneuploid cells increased in a concentration-dependent manner after chronic exposure to lead chromate. Moreover, chronic exposure to lead chromate induced BEP2D cell transformation. Transformed BEP2D cells developed through a series of sequential steps, including altered cell morphology, loss of cell contact inhibition and anchorage-independent growth. Specifically, a 5-day exposure to lead chromate induced foci formation with 0, 1, 5, and 10 $\mu\text{g}/\text{cm}^2$ lead chromate inducing 0, 7, 3, and 15 foci in 10 dishes. Anchorage independence was observed in cell lines derived from these foci. These foci-derived cells also showed centrosome amplification and increases in aneuploid metaphases. Our study demonstrates that particulate Cr(VI) is able to transform human bronchial epithelial cells, and that chromosome instability may play an important role in particulate Cr(VI)-induced neoplastic transformation.

Keywords: lead chromate; neoplastic transformation; bronchial epithelial cells

Hexavalent chromium (Cr(VI)) is a potent bronchial carcinogen with widespread human exposure. Epidemiologic studies show strong associations between Cr(VI) and the development of lung cancer in populations in Japan, Great Britain, West Germany, and the United States (1). These studies suggest—and toxicology studies confirm—that the water-insoluble or “particulate” compounds pose the greatest carcinogenic risk (2, 3). These particulates deposit and persist in the respiratory tract, where they dissolve extracellularly, providing a chronic exposure to soluble Cr(VI) ions (4, 5). These chromate ions enter the cell by facilitated diffusion and are rapidly reduced to trivalent chromium (Cr(III)) along with several short-lived highly reactive intermediates. Then one or some combination of these metabolic products causes a spectrum of genotoxic damage, leading to genomic instability mediated by centrosome amplification and disruption of the spindle assembly checkpoint (6, 7).

(Received in original form February 23, 2007 and in final form June 8, 2007)

This work was supported by NIEHS grant ES10838 (J.P.W.) and the Maine Center for Toxicology and Environmental Health.

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Am J Respir Cell Mol Biol Vol 37, pp 544–552, 2007

Originally Published in Press as DOI: 10.1165/rcmb.2007-0058OC on June 21, 2007

Internet address: www.atsjournals.org

CLINICAL RELEVANCE

This is the first article to show neoplastic transformation by hexavalent chromium in human lung cells. It provides important information for better understanding of the mechanisms involved in human bronchial carcinogenesis induced by particulate chromate.

In addition to the release and uptake of chromate ions, lead (Pb) ions can also enter the cell and undissolved lead chromate particles are internalized by the cell (8–10). The extracellularly released Pb ions or internalized particles play no role in the genotoxic mechanism of lead chromate, though they cannot be completely ruled out of the full carcinogenic mechanism (10–12). For example, more chronic exposures (120 h) of cultured bronchial cells to particulate lead chromate induce persistent chromosome damage and chromosome instability, but in contrast, there is less damage after chronic exposure to soluble sodium chromate (13). It is possible that these effects represent the cation (e.g., Pb ions) inhibiting DNA repair leading to persistent damage, which would be consistent with studies that show that Pb ions can inhibit DNA repair (14, 15), though such a mechanism has not been demonstrated after particulate Cr(VI) exposure or with DNA double-strand break repair. Nevertheless, these data support the observations that particulate chromates are the more potent carcinogens and need to be better understood.

While much is known about the physico-chemical mechanism of particulate Cr(VI)-induced carcinogenesis, much less is understood about the oncogenic changes that occur. The use of lung tumor tissues from Cr(VI)-exposed workers to identify consistent cellular and molecular changes is complicated by severely restricted access to these tissues due to many ongoing lawsuits and by the fact that many workers are also smokers. However, the limited work done (less than 10 tumors in a couple of studies) indicates that the model of clonogenic expansion of mutated cells may not apply to Cr(VI)-induced tumors as common oncogenes such as Ras appear unaffected (16). Instead, particulate Cr(VI) appears to induce chromosome instability (CIN) as an early event in its carcinogenic mechanism. Indeed, particulate Cr(VI) can induce severe aneuploidy as soon as 48 hours after exposure by causing centrosome amplification (6, 7).

Since insufficient numbers of tumors can be obtained, the next best tool is to evaluate human bronchial epithelial cells that have been neoplastically transformed by particulate Cr(VI) to assess the key changes that lead to malignancy. Cr(VI) is known to be genotoxic to both human bronchial fibroblasts and epithelia inducing both structural and numerical chromosomal changes that are consistent with the genomic instability phenotype seen in human bronchial cancers (11, 17), but so far no studies have reported attempts at transforming these cells.

Studies of Cr(VI)-induced transformation in rodent cells show that Cr(VI) can induce both morphologic and neoplastic transformation of cultured rodent cells, but studies in primary human skin cells show that Cr(VI) cannot induce foci formation in these cells (18–22). However, all of these studies, both human and rodent, were very limited in their consideration of molecular and cellular changes, as most were seeking a positive/negative answer and not mechanistic considerations. In addition, none of these studies considered CIN or centrosome amplification in the transformed cells.

This absence of any consideration of genomic instability markers represents a key gap in our understanding of Cr(VI)'s carcinogenic mechanism. Human lung tumors are typically characterized by structural chromosomal instability, a specific type of genomic instability that produces chromosomal abnormalities including deletions, duplications, rearrangements, and unbalanced translocations and significant alterations in chromosome number. Seventy to eighty percent of malignant lung tumors exhibit a complex karyotype with severe aneuploidy, often a triploid to tetraploid complement of chromosomes (23–27). We showed that particulate Cr(VI) induces chromosome instability manifested as aneuploidy in cultured human bronchial cells (6, 7), but no study has yet assessed the role of aneuploidy in Cr(VI)-induced lung tumor tissue or its transformation of cultured cells.

Thus, for a better understanding of the cellular and molecular mechanisms involved in human bronchial carcinogenesis induced by Cr(VI), we focused on the potent carcinogenic form, particulate chromate, and assessed whether it could induce transformation in human bronchial cells and determined if the resultant transformed cells exhibited a phenotype with amplified centrosomes and CIN.

MATERIALS AND METHODS

Chemicals and Reagents

Lead chromate, colcemid, and potassium chloride (KCl) were purchased from Sigma Chemical (St. Louis, MO). Giemsa stain was purchased from Biomedical Specialties Inc. (Santa Monica, CA). Crystal violet, methanol, and acetone were purchased from J. T. Baker (Phillipsburg, NJ). LHC8 culture medium, HBS, E-PET, SBT-I, and DNase were purchased from Biosource (Camarillo, CA). Gurr's buffer was purchased from Invitrogen Corporation (Grand Island, NY). Anti- γ -tubulin (clone GTU-88), anti- α -tubulin-FITC conjugate antibody, and Triton X-100 were purchased from Sigma/Aldrich (St. Louis, MO). Alexa Fluor 555 goat anti-mouse IgG, DAPI, and Prolong were purchased from Molecular Probes (Eugene, OR). Tissue culture dishes, flasks, and plasticware were purchased from Corning Inc. (Acton, MA).

Cells and Cell Culture

The human papillomavirus (HPV)-immortalized human bronchial epithelial cell line, BEP2D, is a suitable model for studying the stages in human bronchial carcinogenesis. These cells have a near diploid karyotype and are nontumorigenic, but can be transformed to a tumorigenic phenotype by agents such as asbestos or α particles (28–30). These cells were routinely cultured in LHC8 medium. Cells were maintained as adherent subconfluent monolayers by feeding at least twice a week and subculturing at least once a week using E-PET and SBT-I. All experiments were conducted on logarithmically growing cells.

Preparation of Cr(VI) Compounds

Lead chromate (CAS# 7758–97–6, ACS reagent minimum 98% purity) was used as a model particulate Cr(VI) salt, and suspensions of lead chromate particles in acetone were prepared and administered as previously described (9). Dilutions were maintained as a suspension using a vortex mixer and treatments were dispensed into cultures directly

from these suspensions. The size of the particles ranged from 0.4 to 58.4 μm , with a mean value of 2.7 μm (10).

Cytotoxicity Assays

Cytotoxicity was determined by a clonogenic assay, which measures the reduction in plating efficiency in treatment groups relative to the controls as previously described (17). There were four dishes per treatment group, and each experiment was repeated at least three times.

Clastogenicity Assay

Chromosome damage analysis. Chromosome damage was measured as the amount of chromosomal aberrations in treatment groups and controls exactly as previously described (17). One hundred metaphases per data point were analyzed in each experiment. Aberrations were pooled as described by Wise and coworkers (9). Each experiment was repeated at least three times.

Aneuploidy analysis. Aneuploidy was determined by counting the number of chromosomes in solid stained metaphases based on our published methods (6). Cells were grouped based on chromosome number into less than 44 chromosomes, diploid (44–48), between 49 and 91 chromosomes, 92 chromosomes, or more than 92 chromosomes. Preparation of chromosomes was done as described above. Each experiment was repeated at least three times.

Centrosome Analysis

Centrosomes were prepared as described previously (6). A monolayer of transformed cells was seeded onto chamber slides. Cells were washed twice in a microtubule stabilizing buffer, fixed with -20°C methanol for 10 minutes, and rehydrated with 0.05% Triton X-100 for 3 minutes. Cells were then incubated in blocking buffer for 30 minutes followed by 1 hour of incubation with a primary anti- γ -tubulin antibody (T-6557; Sigma). Cells were washed four times with PBS and then incubated with Alexa Fluor 555 goat anti-mouse IgG secondary antibody for 1 hour in the dark. Cells were washed four times in PBS and then incubated with a anti- α -tubulin-FITC conjugated antibody for 1 hour in the dark followed by four washes with PBS. A post-fix was performed using 4% paraformaldehyde followed by two PBS washes for 3 minutes each. DNA was stained with DAPI for 30 minutes and then washed once with water. Coverslips were mounted with Prolong and cells were analyzed using fluorescence microscopy. One hundred mitotic cells and 1,000 interphase cells were analyzed per concentration.

Transformation of BEP2D Cells with Lead Chromate

Seventy thousand to 500,000 cells were seeded in 60-mm dishes to achieve an approximately equal cell concentration after treatment. Cultured cells were seeded with five dishes for each concentration and exposed to different concentrations of lead chromate for 5 days. After treatment, cells were then washed twice with PBS to remove undissolved lead chromate particles and then fed with fresh culture medium. Cells were split when they were 80% confluent and reseeded into 10 dishes per concentration. Cells were cultured until foci were found or a maximum of 10 passages. Foci that formed in culture dish were cloned with cloning cylinders. Foci were inspected and desired foci were circled. Growth medium was removed from the dish and cells washed with PBS. The thicker edge of the cloning cylinder was dipped into sterile silicone grease and applied around the selected foci by pressing it lightly against the dish. Forty to 50 μL trypsin was added for 2 to 3 minutes. Foci cells were then transferred to culture dishes.

Anchorage Independent Growth

Anchorage independence assay is considered the most stringent assay for detecting transformation of cultured cells. To determine anchorage-independent cell growth, control and treated cells were suspended in 0.35% agar, plated onto a 0.6% base layer in a 60-mm dish at a density of 5×10^4 , and grown for 4 weeks (31). Cultures were examined microscopically 24 hours after plating to confirm an absence of large clumps of cells. Colonies were visualized by 5% 4-Nitro blue

tetrazolium chloride staining. When cultures were established from anchorage-independent colonies, soft-agar colonies were plucked under sterile conditions and then dispersed in trypsin and replated into culture dishes.

Statistical Analysis

Where mentioned, values are shown as means $\pm$ SEM. Since the percentage calculated in repeated experiments at each treatment level are considered to be independent binomial measurements that can be approximated by a normal distribution, the standard independent two-sample *t* test is valid to test the significance of differences between groups. It is expected that the variances of measurements at different treatment levels are different. We choose to use Satterthwaite's approximated *t* test, which assumes unequal variances between the two groups ($P < 0.05$ was considered significant).

RESULTS

Chronic Exposure to Lead Chromate Causes Cytotoxicity and CIN

To evaluate the cytotoxicity of long-term lead chromate exposure and determine if this chronic exposure induces CIN, manifested as aneuploidy, we treated BEP2D cells with varying concentrations of lead chromate for 120 hours. We found that lead chromate concentrations of 1, 5, and 10 $\mu\text{g}/\text{cm}^2$ induced 74, 60, and 46% relative survival, respectively (Figure 1). These doses also damaged 6.7, 10.7, and 13.7% of metaphases and induced 9, 11.6, and 15 total lesions, respectively (Table 1). The chromosome damage we observed included chromatid lesions, isochromatid lesions, chromatid exchanges, chromosome rings, dicentric, double minutes, acentric fragments, and centromere spreading (Table 2). The primary chromosome damage was chromatid lesions with 1, 5, and 10 $\mu\text{g}/\text{cm}^2$ inducing 4.3, 6.3, and 8.7 chromatid lesions, respectively. Chronic exposure to lead chromate also induced aneuploidy. For example, a 120-hour exposure to 1, 5, and 10 $\mu\text{g}/\text{cm}^2$ lead chromate induced 54.6, 67.0, and 54.9% aneuploid metaphases, respectively (Table 3). More specifically, chronic exposure to these concentrations induced a concentration-dependent increase in the percentage of hypodiploid metaphases (< 46 chromosomes) and corresponding decrease in the percent of diploid metaphases (Table 3).

Particulate Chromate Induces Foci Formation in BEP2D Cells

Foci formation is an established phenotype for loss of contact inhibition and morphologic transformation in cultured cells (32). Adherent cells like epithelia normally maintain contact inhibition in culture. They spread out across the culture flask, and when full the cells stop growing. BEP2D cells routinely exhibit this phenotype. By contrast, loss of contact inhibition manifests itself when cells in a monolayer culture pile up on top of one another to form a focus of growth. We found that a 5-day exposure to lead chromate induced foci formation with 0, 1, 5, and 10 $\mu\text{g}/\text{cm}^2$ lead chromate inducing 0, 7, 3, and 15 foci in 10 dishes (Figure 2 and Table 4). The morphology of untreated and lead chromate-treated BEP2D cells is shown in Figure 2. Untreated BEP2D cells exhibit a flat epithelial-like morphology and appear to grow in a monolayer. However, after treatment with lead chromate, foci appeared within 5 weeks (Figure 2). The morphology of the foci was distinctly different from the surrounding cells. The foci exhibited massive piling up into a virtually opaque multilayer; however, the foci did not resemble the foci described for C3H10T1/2 cells (21) and thus could not be classified into the Type II and Type III foci as previously done for those cells.

Figure 1. The cytotoxicity of lead chromate in human lung epithelial cells (BEP2D). BEP2D cells were treated with lead chromate for 120 hours and induced concentration-dependent cytotoxicity. Data represent an average of three experiments $\pm$ SEM.

Foci-Derived Cells Are Anchorage Independent

Most cell types including BEP2D exhibit anchorage-dependent growth and require a surface on which to attach, flatten out, and divide. By contrast, transformed cells lose this property and are able to grow when suspended in a viscous fluid or gel such as soft agar. Anchorage independence correlates strongly with tumorigenicity and invasiveness in several cell types, such as small-cell lung carcinoma (33). We next considered the ability of these foci-derived cell lines to grow in soft agar. All foci were able to grow well in soft agar, indicating that these cells were indeed anchorage independent (Figure 3 and Table 4).

Foci-Derived Cells Exhibit CIN

We wanted to know if the chromosome instability changes reflected a persistent phenotypic change, so we investigated whether the transformed foci cell lines maintained an aneuploid phenotype. Nine foci cell lines and a control were analyzed for aneuploidy. Eight out of 9 foci showed increases in the percent of aneuploid metaphases compared with nontreated BEP2D cells (32.5%) (Figure 4, Table 5). The majority of these aneuploid cells had less than 46 chromosomes, consistent with our observations that aneuploidy was induced immediately after 120 hours of exposure, and indicating that the change in phenotype was persistent.

Foci-Derived Cells Exhibit Centrosome Amplification

Centrosome amplification is commonly observed in cancer, including lung cancer, and recently we showed that the mechanism of particulate chromate-induced chromosome instability in human lung cells involves centrosome amplification (6). Therefore, we determined whether the transformed cell lines had altered centrosome number. Normal interphase cells have one or two centrosomes. Aberrant interphase cells have more than two centrosomes, as illustrated by the lead chromate-treated cell in Figure 5. Lead chromate-transformed BEP2D

TABLE 1. CHRONIC EXPOSURE TO LEAD CHROMATE INDUCES CHROMOSOME LESIONS

Lead Chromate ($\mu\text{g}/\text{cm}^2$)	Percent of Metaphase with Chromosome Damage	Total Chromosome Damage
0*	5.8 $\pm$ 1.2	6.8 $\pm$ 1.4
1	6.7 $\pm$ 2.3	9.0 $\pm$ 4.2
5	10.7 $\pm$ 5.4	11.6 $\pm$ 5.2
10	13.7 $\pm$ 3.0†	15.0 $\pm$ 4.0†

Average of at least three experiments ($\pm$ SEM) is shown.

* 0: vehicle control (acetone).

† Significant ($P < 0.05$) increase compared with vehicle control.

TABLE 2. TYPES OF CHROMOSOME DAMAGE INDUCED BY LEAD CHROMATE

Lead Chromate ($\mu\text{g}/\text{cm}^2$)	Chromosome Damage							
	Chromatid Lesion	Isochromatid Lesion	Chromatid Exchange	Ring	Dicentric	Double Minute	Acentric Fragment	Centromere Spreading
0*	3.4 ± 0.4	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	1.6 ± 0.7	1 ± 0.5	0.3 ± 0.2	0 ± 0
1	4.3 ± 1.7	1.7 ± 0.7	0 ± 0	0 ± 0	0.3 ± 0.3	1 ± 0.6	0.5 ± 0.4	2 ± 1.6
5	6.3 ± 2.7	2 ± 1.5	0.3 ± 0.3	0.3 ± 0.3	0 ± 0	2.7 ± 1.7	0 ± 0	0 ± 0
10	$8.7 \pm 1.3^\dagger$	1.7 ± 0.9	0 ± 0	0 ± 0	1.7 ± 1.2	2.7 ± 1.2	0.5 ± 0.4	0 ± 0

Average of at least three experiments ($\pm$ SEM) is shown.

* 0: vehicle control (acetone).

† Significant ($P < 0.05$) increase compared with vehicle control.

cells showed increases in the number of interphase cells with more than two centrosomes (Table 6). Three out of 10 foci showed increases in aberrant centrosome number in cells.

Normal mitotic cells have two centrosomes located on opposite poles that produce bipolar spindles and organize the chromosomes along the metaphase plate, ensuring proper chromatid segregation (Figure 5A). In contrast, Figures 5B and 5C show aberrant mitosis with numerous centrosomes, multipolar spindle assembly, and disorganized chromosome alignment and segregation in transformed BEP2D cells by lead chromate. Seven out of 10 foci showed centrosome amplification in mitotic cells (Table 7). The majority of these aberrant mitotic cells had three or four centrosomes.

DISCUSSION

Epidemiologic, animal, and *in vitro* studies have indicated that Cr(VI) is carcinogenic (34, 35). The solubility of Cr(VI) is an important factor in its carcinogenesis. The insoluble Cr(VI) compounds are the most potent carcinogens.

To better understand the genotypic and phenotypic changes associated with particulate chromate-induced lung cancer in humans, it would be ideal to use human bronchial cells to assess the various stages of transformation that lead to malignancies. However, no primary human cell model is currently available for this area of study because such cells have proven to be refractory to malignant transformation *in vitro* (36). The present study is the first to demonstrate that lead chromate-induced malignant transformation of human lung epithelial cells.

Several studies have shown that particulate Cr(VI) induces neoplastic transformation in rodent cells (18, 20, 21); however, only a few studies considered human cell models and none have

considered human bronchial cells (37, 38). One study found that lead chromate induces morphologic transformation in human osteosarcoma cells (37). However, in that study, cells were treated with lead chromate for 24 hours and then washed, and the same concentration of lead chromate was added to cells again for another 24-hour treatment. This procedure was repeated three times. As lead chromate is highly insoluble, and it is not possible to be totally washed away from cells, the actual treatment concentration would effectively be much higher than intended. Furthermore, the cells are already tumor cells and thus events in them may not be relevant to normal human lung cells.

Previous studies indicate that particulate Cr(VI) can induce foci formation and anchorage independence in rodent cells (21), but cannot induce foci formation in normal human fibroblasts (38). Compared with the rodent cell transformation systems, such as C3H/10T1/2 mouse embryo cells (1), lead chromate was a more potent inducer of transformation in BEP2D cells. The reason for this difference may be due to differences in chromate metabolism between rodent and human cells, or differences in DNA lesion and/or their repair between these cells. Indeed, lead chromate induces different cytotoxicity between these cells, with $4 \mu\text{g}/\text{cm}^2$ ($50 \mu\text{M}$) resulting in 9% survival in C3H/10T1/2 cells (21), but $5 \mu\text{g}/\text{cm}^2$ resulting in 53% survival in BEP2D cells. It should be noted that the most biologically effective concentration is one that will induce the most genotoxic damage without killing the cell.

Alterations in centrosome number or function are frequent in transformed cells (39, 40). Because centrosomes dictate the formation of a bipolar mitotic spindle, which is needed for the proper segregation of duplicated chromosomes, aberrancies in centrosome number/function would result in unbalanced chromosome partitioning (41). Consistently, we observed centrosome amplification in lead chromate-transformed BEP2D cells. Cells

TABLE 3. CHRONIC EXPOSURE TO LEAD CHROMATE INDUCES CONCENTRATION-DEPENDENT INCREASE IN ANEUPLOID CELLS

Lead Chromate ($\mu\text{g}/\text{cm}^2$)	Percent of Metaphases					Percent of Aneuploid Metaphases*
	$< 2N^\dagger$	2N	$> 2N$ to $< 4N$	4N	$> 4N$	
0 ^d	16.7 ± 4.5	57.1 ± 6.0	21.4 ± 5.9	1.5 ± 0.5	1.0 ± 0.3	32.5 ± 9.1
1	$27.6 \pm 6.8^\ddagger$	$40 \pm 5.7^\S$	25.4 ± 7.6	0.7 ± 0.4	1.0 ± 0.3	$54.6 \pm 6.9^\ddagger$
5	$43.5 \pm 9.1^\ddagger$	$26.4 \pm 7.6^\S$	22.3 ± 3.9	0.6 ± 0.6	0.7 ± 0.5	$67.0 \pm 8.5^\ddagger$
10	$45.0 \pm 12.5^\ddagger$	$37.7 \pm 8.7^\S$	8.3 ± 3.2	0.7 ± 0.2	0.9 ± 0.9	$54.9 \pm 13.1^\ddagger$

Average of at least three experiments ($\pm$ SEM) is shown.

* Chromosome number other than 46.

† $N = 23$.

‡ Significant ($P < 0.05$) increase compared with vehicle control.

§ Significant ($P < 0.05$) decrease compared with vehicle control.

Figure 2. Lead chromate induces loss of cell contact inhibition in BEP2D cells. This figure shows representative normal BEP2D cells (1) and foci produced after exposure to 5 or 10 $\mu\text{g}/\text{cm}^2$ lead chromate for 5 days (2–6). These foci grew in large masses on the top of the monolayer with complex borders. Foci were ring cloned, subcloned, and tested for growth in agar (Table 1). Magnification is $\times 100$.

with multiple centrosomes tend to form multipolar spindles, which can result in abnormal chromosome segregation during mitosis. It has therefore been postulated that centrosome aberrations may compromise the fidelity of cell division and cause chromosome instability (42). Our previous study showed that lead chromate induced chromosome damage in human lung epithelial cells; that spectrum of damage could reasonably be postulated to induce the type of rearrangements associated with neoplasia (43). We also showed that particulate Cr(VI) induces CIN through centrosome amplification in human lung fibroblasts. In addition, lead chromate induced DNA double strand breaks in human lung fibroblasts, a type of damage that can lead to genetic instability and tumorigenesis (10).

BEP2D cells treated with lead chromate for 5 d showed an increase in aneuploidy, and this phenotype persisted in foci-derived cells even after 10 passages in culture, indicating that it was a permanent change. This finding is consistent with our previous data showing that 120 hours of exposure to lead chromate in human bronchial fibroblasts induced a persistent aneuploid phenotype (6, 7). In cancer, centrosomes are often found amplified to greater than two per cell, and these tumor cells frequently have aneuploid genomes (44). Karyotypic alterations (including whole chromosome loss or gain), ploidy changes, and a variety of chromosome aberrations are common in cancer cells. Indeed, it has long been recognized that errors in the centrosome duplication cycle may be an important cause of aneuploidy and thus contribute to cancer formation.

The data demonstrate the importance of centrosome amplification in Cr(VI)-induced transformation. The mechanisms underlying Cr(VI)-induced centrosome amplification are uncertain. It may involve an effect of Cr(VI) directly on centrosome duplication, maturation, or separation pathways; however,

a chemical carcinogen has not yet been shown to disrupt these pathways. Another interesting possibility is that the underlying lesion may be DNA double strand breaks. We have recently shown that Cr(VI) can induce DNA double strand breaks, and recent studies have demonstrated links between DNA damage and centrosome amplification numerical aberrations. DNA damage can cause a disruption of centrosome function and loss of the damaged nucleus by mitotic catastrophe (45). Defects in a number of key genes involved in DNA damage repair have been shown to cause aberrations in centrosome number (46–48), further implicating DNA damage in the generation of centrosome amplification. Our previous study showed that lead chromate can cause DNA double strand breaks and can induce S and G2 phase arrest (12). Because the centrosome cycle is controlled by an intrinsic timing mechanism, if cells are arrested for an abnormally long time in G2, then the centrosome cycle might resume even though mitosis has not been traversed. Thus, it is possible that chronic exposure to Cr(VI) may result in a prolonged cell cycle arrest, causing uncoupling of the cell cycle from centrosome duplication and resulting in centrosome amplification.

We did not observe a clear concentration-dependent response in transformation. This is consistent with previous studies of lead chromate-induced transformation in rodent cells, which also did not see a concentration response for particulate Cr(VI)-induced transformation (21). We think that there may be two different mechanisms occurring that explain these results. Specifically, we observed that the 5 $\mu\text{g}/\text{cm}^2$ concentration exhibited a lower

TABLE 4. TRANSFORMATION EFFICIENCY OF BEP2D CELLS

Lead Chromate ($\mu\text{g}/\text{cm}^2$)	Foci Frequency (total foci/10 dishes)	Foci Growth in Soft Agar
0	0/10	ND
1	7/10	+*
5	3/10	++ ^c
10	15/10	++ [†]

Definition of abbreviation: ND, not done as there were no foci.
* Colonies were comparatively small and few in number.
[†] Colonies were comparatively large and numerous.

Figure 3. Lead chromate induces anchorage independent growth in BEP2D cells. This figure shows representative photographs illustrating that cells grow colonies on soft agar. (A) Magnification ($\times 100$) of a cell colony growing in soft agar. (B) Colonies were visualized by 5% 4-Nitro blue tetrazolium chloride staining.

Figure 4. Representative examples of aneuploid phenotype in foci-derived BEP2D cells. This figure shows representative examples of normal and aberrant mitotic figures. (A) This picture represents a normal mitotic cell with 46 chromosomes. (B) This picture represents an aberrant mitotic cell with 67 chromosomes. (C) This picture represents an aberrant mitotic cell with 34 chromosomes.

response than the 1 $\mu\text{g}/\text{cm}^2$ dose. The response at the 10 $\mu\text{g}/\text{cm}^2$ concentration was higher than the response at either of the lower two concentrations. However, while the lowest concentration, 1 $\mu\text{g}/\text{cm}^2$, did induce foci formation, the foci only grew weakly in soft agar. By contrast, the foci produced at the two higher concentrations grew well in soft agar. Thus, we suggest that the foci at the lowest concentration may actually be enhanced growth variants that are only partially transformed, while the foci at the higher concentrations are fully transformed. This possibility is consistent with previous studies of particulate metals, including lead chromate, which showed that these metal particles induced enhanced growth variants, but did not induce full transformation of primary rat tracheal epithelial cells (49, 50). This possibility, albeit untested, is also consistent with our data, as we observe a dose-response from 5 to 10 $\mu\text{g}/\text{cm}^2$ for transformation, with the 1 $\mu\text{g}/\text{cm}^2$ concentration likely representing the peak of an enhanced growth variant effect.

We also observed a lack of a concentration-dependent response with respect to the production of chromosome aberrations. Previously, we demonstrated that lead chromate did induce a concentration-dependent response in these cells after a 24-hour exposure (43). Thus, the lack of a dose-response seen here after a 24-hour exposure probably reflects an increase in cells with aberrations undergoing apoptosis, or an induction in DNA repair preventing an increase in lesions. Notably, compared with the 24-hour exposure, we found an increase in the formation of more complex chromosome aberrations with the

spectrum expanding from only chromatid/isochromatid lesions to chromatid exchanges, chromatid rings, double minutes, acentric fragments, and centromere spreading at the longer exposure time.

It is unclear if particulate Cr(VI) would induce transformation in primary human lung cells. Typically, these cells do not survive in culture long enough for transformation studies, and historically fully competent human cells have resisted chemical transformation. Thus, it cannot be ruled out that the loss of functional p53 in these cells plays a role. Cr(VI)-induced tumors have been shown to have p53 mutations, and studies of cells in culture show that p53 is a target for Cr(VI) (41–53). Cyclin/cdk and p53-mediated cell cycle pathways regulate centrosome homeostasis by ensuring the integrity of the G1/S and G2/M cell cycle checkpoints. Loss of p53 function alone is not sufficient to cause the development of centrosome amplification, but its loss combined with failure of the G1/S checkpoint activation after genotoxic stress can induce centrosome over-duplication (54). Thus, it is possible that Cr(VI)-induced tumorigenesis may involve loss of p53 function in the presence of DNA damage, leading to centrosome amplification.

The mechanism for particulate chromate-induced genotoxicity indicates that particles dissolve outside the cell and enter the cell as their respective ions and that once inside the cell, the chromate ions are reduced to Cr(III) through a series of redox reactions releasing Cr(V), Cr(IV), and free radicals as intermediates (55, 56). Cr(III), one of the intermediates, or some

TABLE 5. CHROMOSOME INSTABILITY IN FOCI-DERIVED BEP2D CELLS

Lead Chromate ($\mu\text{g}/\text{cm}^2$)	Foci Cell Line	Percent of Metaphases					Percent of Aneuploid Metaphases [†]
		< 2N*	2N	> 2N to < 4N	4N	> 4N	
1	F1	35.3	50.4	10.8	0.7	0.7	47.5
	F3	34.5	53.5	7.7	1.4	0	43.7
	F4	59.3	36.2	2.8	0	1.1	63.3
5	F1	47.5	49.7	2.2	0	0	49.7
	F2	21.9	58.1	4.5	1.3	0.6	28.4
	F3	38.3	51.9	5.8	1.3	0	45.5
10	F1	40.3	55.2	1.3	0	0.6	42.2
	F3	40.4	55.9	1.2	0	1.2	42.8
	F5	43.5	54.2	2.4	0	0	45.8

* N = 23.

[†] Chromosome number other than 46.

Figure 5. Representative examples of normal interphase and aberrant mitotic with centrosome amplification. This figure shows representative example of aberrant interphase and mitotic cells from foci-derived BEP2D cells. *Blue* (DAPI) represents the DNA, *green* (FITC) represents the microtubules, and *red* (Alexa 555) represents the centrosomes. (A) This picture represents a normal interphase cell with one centrosome (*red dot*). (B) This cell is an aberrant interphase cell with five centrosomes. (C) This cell is an aberrant mitotic figure with four centrosomes, multipolar spindle fibers, and disorganized DNA. (D) This picture represents an aberrant mitotic cell with four centrosomes that is dividing into three daughter cells.

combination of them induce DNA double strand breaks and centrosome amplification, leading to structural and numerical chromosomal changes (6, 10). It is possible that the full carcinogenic mechanism may include the genotoxic mechanism and an epigenetic mechanism with the divalent cation (e.g., lead), inhibiting the repair of the Cr(VI)-induced damage, which could lead to increased chromosome instability. Although our data suggest that internalized particles have no apparent effect, the cells with internalized particles are capable of condensing chromosomes and undergoing mitosis (10), which indicates that the

particles may have the potential for damaging chromosomes in a physical manner as proposed for asbestos (57). However, we have looked at particles inside human bronchial cells after several days of exposure and always find them sequestered in vacuoles (data not shown). Further work is aimed at elucidating the ability of the divalent cation to inhibit the repair of Cr(VI)-induced damage and determining changes in genes related to chromosome instability in these transformed cell lines, including genes involved in centrosome amplification and the spindle assembly checkpoint.

TABLE 6. CENTROSOME AMPLIFICATION IN PERCENT OF INTERPHASE OF FOCI-DERIVED BEP2D CELLS

Lead Chromate ($\mu\text{g}/\text{cm}^2$)	Foci Cell Line	Centrosome Number									
		1	2	3	4	5	6	7	8	> 2	
0	Ø	12.7	85.6	1.1	0.3	0.1	0.1	0	0.1	1.7	
1	F1	13.7	77.8	4	3.1	1.1	0.2	0	0.1	8.5	
	F2	15.3	76.6	4.2	2.9	0.5	0.1	0.3	0.1	8.1	
	F5	14.6	81.9	1.8	1.7	0	0	0	0	3.5	
5	F1	13.2	84.4	1.1	0.8	0.3	0.2	0	0	2.4	
	F2	14.6	81.9	1.4	1.8	0.3	0	0	0	3.5	
10	F1	14.9	83.1	1.4	0.4	0.1	0.1	0	0	2	
	F2	14.4	77.6	3.4	4.2	0.4	0	0	0	8	
	F3	13.3	85.9	0.4	0.3	0.1	0	0	0	0.8	
	F4	14.4	84	1	0.3	0.1	0.1	0.1	0	1.6	
	F5	14.2	84.4	0.9	0.4	0.1	0	0	0	1.4	

TABLE 7. CENTROSOME AMPLIFICATION IN MITOTIC CELLS OF FOCI-DERIVED BEP2D

Lead Chromate ($\mu\text{g}/\text{cm}^2$)	Foci Cell Line	Centrosome Number							
		2	3	4	5	6	7	8	> 2
0	Ø	98	1	1	0	0	0	0	2
1	F1	82	3	11	2	2	0	0	18
	F2	72	6	17	4	1	0	0	28
	F5	90	2	8	0	0	0	0	10
5	F1	90	4	3	2	1	0	0	10
	F2	92	6	1	1	0	0	0	8
10	F1	99	1	0	0	0	0	0	1
	F2	81	0	17	1	0	0	1	19
	F3	97	3	0	0	0	0	0	3
	F4	89	4	5	1	1	0	0	11
	F5	97	3	0	0	0	0	0	3

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Curtis C. Harris, National Cancer Institute, and Tom K. Hei, Columbia University, for the BEP2D cells. They also thank David Kirstein for administrative support.

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