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Human Skin Cells Are More Sensitive than Human Lung Cells to the Cytotoxic and Cell Cycle Arresting Impacts of Particulate and Soluble Hexavalent Chromium

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Abstract

Hexavalent chromium Cr(VI) is a known human lung carcinogen, with solubility playing an important role in its carcinogenic potency. Dermal exposure to Cr(VI) is common and has been associated with skin damage; however, no link between chromate exposure and skin cancer has been found. In this study, we compared the cytotoxic and clastogenic effects of Cr(VI) and its impacts on cell cycle progression in human lung and skin fibroblasts. We found human skin cells arrested earlier in their cell cycle and exhibit more cytotoxicity than human lung cells, despite taking up similar amounts of Cr. These outcomes are consistent with a hypothesis that different cellular and molecular responses underlie the differences in carcinogenic outcome in these two tissues.

Keywords

Chromium; genotoxicity; cytotoxicity; chromate; skin; lung; hexavalent chromium

Introduction

Hexavalent chromium (Cr(VI)) is a known human lung carcinogen[1–4]. Epidemiological studies performed on workers exposed to Cr(VI) indicate that chromate exposure increases the risk of developing lung cancer 2–80 fold [1]. Exposure to Cr(VI) has also been associated with skin irritation, deep ulceration and cytotoxicity [1,2]. However, epidemiological studies have found no link between chromate exposure and skin cancer [1, 2].

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Conflict of Interest

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The mechanism of chromate-induced lung cancer remains unknown, but epidemiological, whole animal and cell culture studies pinpoint particulate Cr(VI) compounds as the most potent human lung carcinogens [2–3,5–8]. The increased carcinogenicity of particulate Cr(VI) compounds may be due to their persistence at human lung bifurcations where chromate-associated cancer occurs[8–10]. Studies of particulate chromate compounds, using lead chromate as a model, have found that particulate Cr(VI) compounds dissolve outside the cell, releasing the chromate anion into the extracellular environment [11–13]. The chromate anion then enters the cells, resulting in chronic exposure to Cr(VI), and it is the chromate anion that is the proximate genotoxic agent [11–14]. Water soluble Cr(VI) compounds are also potent genotoxic compounds, but their carcinogenic potential is weak [2–5, 7], possibly due to their rapid clearance from the cellular microenvironment in the lung [15–16].

Interestingly, despite dermal exposure of workers to chromate, epidemiological studies suggest there is no link between chromate exposure and skin cancer [1]. One recent animal study suggests that chromate exposure through drinking water can increase UV-induced skin cancer, but chromate alone is a weak skin carcinogen [17]. Even though chromate does not appear to induce skin cancer, it does cause skin toxicity including allergic contact dermatitis and skin ulcers in chromium workers [18]. Human skin cells were widely used as an in vitro experimental model to study the potential mechanisms underlying Cr(VI). Studies have shown that Cr(VI) induces cytotoxicity, clastogenicity, DNA double strand breaks and anchorage independence in human skin cells [19–21]. However, the differences in the carcinogenic potential of chromate in the lung and skin remain unknown.

Accordingly, the purpose of this study is to investigate the cytotoxic and genotoxic effects of soluble and particulate Cr(VI) compounds on human skin cells and compare those effects with that of human lung cells.

Materials and Methods

Reagents

Sodium chromate, lead chromate, colcemid, and potassium chloride were purchased from Sigma-Aldrich (St. Louis, MO). Gurr's buffer, trypsin/EDTA, sodium pyruvate, penicillin/streptomycin, and L-Glutamine were purchased from Invitrogen (Carlsbad, CA). Giemsa stain was purchased from Biomedical Specialties (Santa Monica, CA). Sodium dodecyl sulfate (SDS) was purchased from American Bioanalytical (Natick, MA). Crystal violet, methanol and acetone were purchased from J.T. Baker (Phillipsburg, NJ). Dulbecco's minimal essential medium and Ham's F-12 (DMEM/F-12) were purchased from Mediatech (Herndon, VA). Cosmic calf serum (CCS) was purchased from Hyclone (Logan, UT). Tissue culture dishes, flasks, and plasticware were purchased from Corning (Acton, MA).

Cell Culture

WTHBF-6 and BJhTERT cells were used as model human lung and skin cells, respectively. WTHBF-6 cells are hTERT-expressing human lung fibroblasts and BJhTERT are hTERT-expressing human skin fibroblasts. Both cell lines exhibit a diploid karyotype, normal

growth parameters and an extended lifespan. The cells were cultured in a 50:50 mix of Dulbecco's minimal essential medium and Ham's F12 medium plus 15% cosmic calf serum, 1% L-glutamine and 1% penicillin/streptomycin. All cells were maintained in a 37°C, humidified incubator with 5% CO₂. At least once a week, cells were subcultured using 0.25% trypsin/1mM EDTA solution and all experiments were performed on logarithmically growing cells.

Preparation of Chromium Compounds

Sodium chromate is a soluble form of Cr(VI) and was administered as a solution in water as previously described [8]. Lead chromate is a particulate Cr(VI) compound and was administered as a suspension in water, as previously described [8,22].

Intracellular Ion Uptake

Cells were prepared for determination of intracellular Cr levels as previously described [23]. Intracellular Cr concentrations were determined from cell lysates using an inductively coupled plasma optical emission spectrometer (ICP-OES) as previously described [23]. Intracellular Cr concentrations were converted from ug/L to uM by dividing by the volume of the sample, the atomic weight of chromium, the number of cells in the sample and the average cell volume. Each experiment was repeated at least three times.

Cytotoxicity

Cytotoxicity was measured with a clonogenic survival assay using standard methods [8]. Clonogenic survival assays measure the reduction in plating efficiency in treatment groups relative to control dishes. Each treatment group contained four dishes and each experiment was repeated at least three times. The plating efficiency for both human lung and skin fibroblasts was consistently between 10–15% for controls. Each experiment was repeated at least three times.

Chromosome Damage

Clastogenicity was assessed as a measure of chromosome damage using standard methods and scoring criteria[8]. One-hundred metaphases per data point were analyzed in each experiment. Each experiment was repeated at least three times.

Cell Cycle Analysis

Cell cycle analysis was performed according to our published methods [24]. Briefly, after Cr(VI) exposure, cells were harvested, washed once with PBS, and fixed with –20°C 70% ethanol. Cells were allowed to fix overnight and then digested with RNase A and stained with propidium iodide for 30 min. PI intensity was detected using a BD FACSCalibur flow cytometer, and data was analyzed using ModFit LT 3.0 software. Each experiment was repeated at least three times.

Statistics

The Student's t-test was used to calculate p-values to determine the statistical significance of the difference in means. No adjustment was made for multiple comparisons. Interval estimates of differences are 95% confidence intervals, based also on Student's t distribution.

Results

Chromium Ion Uptake in Human Lung and Skin Cells

Both particulate and soluble Cr(VI) produced a similar concentration-dependent increase in intracellular Cr ion levels in human lung and skin cells after 24 h exposure (Figure 1a and 1b). For example, exposure to 5 uM sodium chromate produced an intracellular Cr ion level of 2,740 uM in human lung cells, compared to 1,915 uM in human skin cells, and at 0.5 ug/cm² lead chromate, the intracellular Cr concentrations in human lung and skin cells were 677 and 668, respectively.

Comparative Cytotoxicity of Hexavalent Chromium Compounds in Human Lung and Skin Cells

Both particulate and soluble Cr(VI) induced concentration-dependent increases in cytotoxicity in human lung and skin cells (Figure 2). Skin cells were similarly sensitive to soluble Cr(VI)-induced cytotoxicity as lung cells. Specifically, exposure to 0.5, 1, 2.5 and 5 uM sodium chromate induced 90, 82, 22 and 0 percent relative survival, respectively, in human skin cells compared to 62, 68, 41 and 5 percent in human lung cells (Figure 2a and 2b). For lead chromate, skin cells were more sensitive than lung cells. For example, 0.5 and 1ug/cm² lead chromate induced 25 and 8 percent relative survival in human skin cells, respectively, compared to 67 and 42 percent in human lung cells, respectively (Figure 2b). When we considered the cytotoxicity data based on intracellular levels, the data show that skin cells are actually more sensitive to the cytotoxic effects of Cr(VI) for both compounds. (Figure 2c and 2d).

Comparative Genotoxicity of Hexavalent Chromium Compounds in Human Lung and Skin Cells

Particulate and soluble Cr(VI) induced concentration-dependent increases in genotoxicity in human lung and skin cells (Figures 3 and 4). For soluble Cr(VI) the amount of chromosome damage was similar (Figure 3). For example, 1 uM sodium chromate induced aberrations in 16 percent of metaphases and 18 total aberrations in skin cells, respectively, compared to 19 percent and 24 total aberrations in lung cells (Figures 3a and 3b). This response remained similar after comparing the cells based on intracellular levels (Figures 3c and 3d). Interestingly, 5 uM sodium chromate induced sufficient cell cycle arrest that no metaphases were seen in human lung cells (Figure 3a), but this concentration induced aberrations in 47 percent of metaphases and 68 total aberrations in skin cells indicating a different cell cycle response in the two cell lines.

In contrast to the soluble Cr(VI) results, particulate Cr(VI) was more genotoxic to skin cells than lung cells (Figure 4). For example, 0.5 ug/cm² lead chromate induced aberrations in 34 percent of metaphases and 61 total aberrations in skin cells compared to 20 percent and 25

total aberrations in lung cells (Figures 4a and 4b). 5 ug/cm² lead chromate induced cell cycle arrest and no metaphases were seen in both human lung and skin cells. This difference remained after comparing the cells based on intracellular levels indicating it was not simply a consequence of the amount of Cr inside the cells (Figures 4c and 4d).

Comparative Cell Cycle Arrest Induced by Hexavalent Chromium Compounds in Human Lung and Skin Cells

Both particulate and soluble Cr(VI) induced cell cycle arrest in human skin cells. For soluble Cr(VI), the arrest in skin cells was in G1. At 2.5 and 5 uM sodium chromate, cells accumulated in G1 with an accompanying decrease of cells in G2/M (Figure 5a). By contrast, in lung cells, soluble Cr(VI) induced cell cycle arrest in G2/M (Figure 5b). More specifically, 2.5 uM sodium chromate induced cell accumulation in both G2/M and S with a concomitant decrease in cells in G1. These data, considered with the observations of reduced metaphases in the genotoxicity analysis, strongly suggest the observed arrest in lung cells is in G2 and not M phase.

Particulate Cr(VI) induced a different and more complex pattern of cell cycle arrest than soluble Cr(VI) (Figures 5 and 6). In skin cells, lower concentrations of lead chromate (0.1–1 ug/cm²) induced S phase arrest and a decrease in cells in G1 and G2, while the highest concentration (5 ug/cm²) induced a G1 arrest with a decrease in cells in S and G2 (Figure 6A). By contrast, particulate Cr(VI) induced a strikingly different pattern of arrest in lung cells compared to skin cells (Figures 6a and 6b). Lower concentrations of lead chromate (0.1–1 ug/cm²) induced G2 phase arrest and a decrease in cells in G1 and little change in cells in S phase, while the highest concentration (5 ug/cm²) induced an S phase arrest with a decrease in cells in G1 and G2 (Figure 6b).

Discussion

Cr(VI) is a known human lung carcinogen with its particulate compounds being the most potent [1,2]. Exposure through direct contact can also cause allergic skin reactions and skin damage [17,18] but the potential to cause skin cancer is still unclear. This study is the first to compare the toxicity of Cr(VI) in human skin and lung cells. Our data found that human skin and lung cells have some very different responses to particulate and soluble Cr(VI) at the cellular level. It is important to note that we cultured the cells in the same culture medium, which rules out artificial extracellular factors in these differences.

The most striking difference was in the cell cycle response. Human skin cells arrested earlier in the cell cycle than lung cells after Cr(VI) treatment. For skin cells, the arrest occurred in G1 compared to G2 in lung cells after soluble Cr(VI) exposure, and occurred in S (low dose) and G1 (high dose) for skin cells compared to G2 (low dose) and S (high dose) in lung cells after particulate Cr(VI) exposure. This outcome suggests that skin cells are detecting damage from Cr(VI) much earlier in the cell cycle and arresting. The underlying trigger for this difference is uncertain, but it is clearly not due to differences in Cr(VI) uptake as both cell types internalized the same amount of Cr.

It may be that skin cells detect some Cr(VI)-induced lesions better than human lung cells. For example, Cr(VI)-induced DNA double strand breaks only occur in G2 phase in lung cells [22]. It is possible that these breaks occur in G1 in skin cells leading to the arrest, but it is currently unknown when these breaks occur in skin cells. Our data showing no difference in chromosome damage between the cell types after soluble Cr(VI) exposure would seem to argue against this particular possibility. Nevertheless, an interesting hypothesis emerges that suggests human skin cells might be resistant to Cr(VI)-induced carcinogenesis because they can detect the underlying lesion earlier in the cell cycle. Such a hypothesis is consistent with reports showing bypass of the G1/S checkpoint result in increased cell proliferation and neoplastic progression [25, 26].

Consistent with this hypothesis, we observed, based on intracellular Cr levels, that Cr(VI) induced more cell death in skin cells than in lung cells. Cell cycle arrest connects with apoptotic pathways in a cell. For example, many DNA damaging agents induce G1 arrest through up-regulation of p53 or down-regulation of cyclin D1, Cdk 2, and Cdk 4 expression leading to subsequently apoptotic cell death [27–29]. It may be that by first arresting in G1, skin cells are able to remove more damaged cells.

The observation that skin cells are more sensitive to Cr(VI) cytotoxicity is consistent with studies showing metals are more cytotoxic to skin cells than lung cells. For example, one study showed silver nanoparticles are more cytotoxic to human skin epithelium carcinoma cells than to human lung carcinoma cells [30]. Another study found immortalized human keratinocyte cells were more sensitive to cobalt-doped tungsten carbide nanoparticle cytotoxicity than a human lung epithelial carcinoma cell line [31]. A third study showed silica-coated CdSe/ZnS quantum dots caused apoptosis/necrosis in human skin fibroblasts but not in lung fibroblasts [32]. A study with primary North Atlantic right whale lung and skin fibroblasts, also reported that skin cells were more sensitive to Cr(VI) cytotoxicity than lung fibroblasts [33]. Thus, this outcome may be a general effect for metals.

It is also interesting to note that in skin cells, more chromosome damage occurred than in lung cells after particulate Cr(VI) exposure. The explanation for the differences in clastogenicity is uncertain. One possibility is the difference is caused by the cation, lead, which maybe more clastogenic to skin cells. Our previous studies in lung cells indicate the cation is not a contributing factor for particulate Cr(VI)-induced cytotoxicity and chromosome damage [11–14]. However, lead can interfere with the repair of DNA damage. One study reported lead inhibits theapurinic/aprimidinic endonuclease (APE1), an important repair protein in the DNA base excision repair pathway [34]. Gastaldo et al., showed lead inhibits the non-homologous DNA end-joining (NHEJ) double strand break repair process by inhibiting DNA-PK activity [35]. Perhaps, skin cells are more sensitive to lead than lung cells, and in skin, lead interferes with the repair of DNA double strand breaks in skin cells resulting in the increase of chromosome breaks. Alternatively, it may be that skin cells are more proficient at lead uptake and simply have more lead in them. Consistent with this possibility, we have found skin cells do take up twice as much lead as lung cells. For example, exposure to 0.5 $\mu\text{g}/\text{cm}^2$ lead chromate results in intracellular lead levels of 191 and 88 μM lead in human skin and lung cells, respectively (unpublished data).

In summary, our data show particulate and soluble Cr(VI) induce cytotoxicity and genotoxicity and cell cycle arrest in both human lung and skin cells. However, there are significant differences in cell cycle and cell death responses that suggest mechanisms where skin cells are more protected than lung cells from Cr(VI) carcinogenicity, which is consistent with observations that Cr(VI) causes lung cancer and damages skin, but does not cause skin cancer. Future work is aimed at determining the mechanisms underlying the differential cell cycle response in these two cell types.

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Fig 1. Human Lung and Skin Fibroblasts Take Up Similar Levels of Cr

This figure shows, after exposure to Cr(VI), human lung and skin cells take up Cr in a concentration-dependent manner. Data represent the average of 3 independent experiments. Error bars = standard error of the mean. * = Significantly different from control. **(a)** Cr uptake after soluble Cr(VI) treatment. **(b)** Cr uptake after particulate Cr(VI) treatment

Fig 2. Particulate and Soluble Cr(VI) Are More Cytotoxic to Human Skin Cells than Human Lung Cells

This figure shows Cr(VI) induces cytotoxicity in human lung and skin cells in a concentration-dependent manner. Correcting for differences in uptake shows skin cells are more sensitive than lung cells. Data represent the average of 3 independent experiments. Error bars = standard error of the mean. * = Significantly different from control ($p < 0.005$). ** = Significantly different between human lung and skin cell lines ($p < 0.05$). (a) Soluble Cr(VI) cytotoxicity based on administered dose. (b) Particulate Cr(VI) cytotoxicity based on administered dose. (c) Soluble Cr(VI) cytotoxicity based on intracellular Cr ion concentration. (d) Particulate Cr(VI) cytotoxicity based on intracellular Cr ion concentration.

Fig 3. Soluble Cr(VI) Induces Similar Genotoxicity in Human Skin and Lung Cells

This figure shows sodium chromate induces similar amounts of chromosome damage in human lung and skin cells in a concentration-dependent manner. Data represent the average of 3 independent experiments. Error bars = standard error of the mean. NM = No metaphases observed. * = Significantly different from control in lung cells ($p < 0.05$). (a) Soluble Cr(VI) genotoxicity based on administered dose and using the percent of metaphases with damage to quantify the effect. (b) Soluble Cr(VI) genotoxicity based on administered dose and using the total damage in 100 metaphases to quantify the effect. (c) Soluble Cr(VI) genotoxicity based on intracellular Cr concentration and using the percent of metaphases with damage to quantify the effect. (d) Soluble Cr(VI) genotoxicity based on intracellular Cr concentration and using the total damage in 100 metaphases to quantify the effect

Fig 4. Particulate Cr(VI) Induces More Genotoxicity in Human Skin Cells than in Human Lung Cells

This figure shows lead chromate induces chromosome damage in human lung and skin cells in a concentration-dependent manner. Correcting for differences in uptake shows skin cells are more sensitive than lung cells. Data represent the average of at least 3 independent experiments. Error bars = standard error of the mean. * = Significantly different from control ($p < 0.05$). *** = Significantly different between human lung and skin cell lines ($p < 0.01$). (a) Particulate Cr(VI) genotoxicity based on administered dose and using the percent of metaphases with damage to quantify the effect. (b) Particulate Cr(VI) genotoxicity based on administered dose and using the total damage in 100 metaphases to quantify the effect. (c) Particulate Cr(VI) genotoxicity based on intracellular Cr concentration and using the percent of metaphases with damage to quantify the effect. (d) Particulate Cr(VI) genotoxicity based on intracellular Cr concentration and using the total damage in 100 metaphases to quantify the effect

Fig 5. Skin Cells Arrest Earlier in the Cell Cycle than Lung Cells after Soluble Cr(VI) Exposure

This figure shows sodium chromate induces cell cycle arrest in human skin and lung cells. Data represent the average of 3 independent experiments. Error bars = standard error of the mean. **(a)** Soluble Cr(VI) induces G1 phase arrest in human skin cells. **(b)** Soluble Cr(VI) induces G2/M phase arrest in human lung cells. * = Significantly different from control ($p < 0.05$). ** = Significantly different from control ($p < 0.005$). *** = Significantly different between human lung and skin cell lines ($p < 0.05$). **** = Significantly different between human lung and skin cell lines ($p < 0.005$).

Fig 6. Skin Cells Arrest Earlier in the Cell Cycle than Lung Cells after Particulate Cr(VI) Exposure

This figure shows lead chromate induces cell cycle arrest in human skin and lung cells. Data represent the average of 3 independent experiments. Error bars = standard error of the mean. **(a)** Particulate Cr(VI) induces S phase (low dose) and G1 phase (high dose) arrest in human skin cells. **(b)** Particulate Cr(VI) induces G2/M phase (low dose) and S phase (high dose) arrest in human lung cells. * = Significantly different from control ($p < 0.05$). ** = Significantly different from control ($p < 0.005$). *** = Significantly different between human lung and skin cell lines ($p < 0.05$). **** = Significantly different between human lung and skin cell lines ($p < 0.005$).