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# Induction of Morphological Changes in BEAS-2B Human Bronchial Epithelial Cells Following Chronic Sub-Cytotoxic and Mildly Cytotoxic Hexavalent Chromium Exposures

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Certain hexavalent chromium (Cr(VI)) compounds are well established occupational respiratory tract carcinogens. However, despite extensive studies, the cellular and molecular mechanisms underlying Cr(VI)-induced lung cancer remain poorly understood. In fact, the models used were often suboptimal and yielded conflicting results that were heavily dependent upon the system and experimental conditions employed. Here, we investigated the effects of chronic subcytotoxic and mildly cytotoxic (0.1–2 μM) Cr(VI) exposures on cultures of human bronchial epithelial cells, the main targets of Cr(VI) carcinogenicity. Our studies with the nontumorigenic BEAS-2B cell line suggest that relatively short exposures (h) to sublethal Cr(VI) doses (0.1–1 μM) may render these cells less sensitive to contact inhibition. We have also observed a reduced sensitivity to Cr(VI)-induced apoptosis shortly after the beginning of exposure to a mildly cytotoxic Cr(VI) dose (2 μM). Further studies are needed to determine whether these two phenotypes are involved in the Cr(VI)-induced carcinogenic process. Additionally, evidence gathered in this study strongly points to a Cr(VI) interference with cell adhesion to the substratum and with cell–cell interactions. Finally, by chronically exposing BEAS-2B cells to mildly cytotoxic Cr(VI) doses (1 and 2 μM), we were able to induce changes in cell morphology and pattern of growth characteristic of an early phase of pre-malignant progression. 

Key words: cellular models; BEAS-2B cells; hexavalent chromium; chronic exposures; transformation

## INTRODUCTION

Certain Cr(VI) compounds extensively used in various industries are well established occupational human lung carcinogens through chronic inhalation [1–3]. These compounds may also pose an environmental health risk, as they are widespread in cigarette smoke, automobile emissions, and landfills [4,5]. Although Cr(VI) itself does not react extensively with DNA, Cr(VI)-containing compounds are genotoxic and can induce a wide variety of DNA lesions, including DNA adducts, DNA strand breaks, oxidized DNA bases, abasic sites and crosslinks, as well as mutations, chromosomal aberrations, sister chromatid exchanges, and microsatellite instability [4,6,7]. In fact, in the presence of intracellular reducing agents such as ascorbate and glutathione, Cr(VI) undergoes rapid metabolic reduction to Cr(III), through relatively unstable Cr(V) and Cr(IV) reactive intermediates [8–13], all displaying reactivity towards DNA [4,14,15]. Moreover, a variety of other reactive species are formed during the oxidation of the Cr(VI) reductants, such as organic radical species, which may also produce DNA damage and, subsequently, mutations and chromosomal damage [4,8]. At the cellular level, Cr(VI) exposure can lead to cell cycle arrest, apoptosis or neoplastic transformation [16–22]. For instance, it has been shown that lead chromate induces morphological and neoplastic transformation of C3H/10T1/2 cells [19,20].

Although our present knowledge of the mechanisms underlying Cr(VI) effects, in particular those

Abbreviations: MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; AFM, Atomic Force Microscopy; NHBE, normal human bronchial epithelial; ECM, extracellular matrix; BEGM, bronchial epithelium growth medium; Cr(VI), hexavalent chromium; SV40, simian virus 40; WT, wild-type; SD, standard deviation.

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that may be causative factors in neoplasia, is still very incomplete, there is strong evidence that they differ from those underlying lung cancers induced by cigarette smoke and asbestos [23–26]. Considering the information available, a reasonable hypothesis is that Cr(VI) mutagenesis involves both a decrease in the fidelity of DNA replication [27–33] and deficiencies in DNA repair mechanisms [26,34–38]. In this context, it must be acknowledged that some cellular studies are probably compromised by the use of inadequate systems and/or of exposure regimens that are not representative of any human exposures associated with the development of this pathology [4,39,40].

Here we describe a study on which multiple events of Cr(VI)-induced carcinogenesis were more closely mimicked by chronically exposing human lung epithelial cells to subcytotoxic and mildly cytotoxic Cr(VI) insults. When BEAS-2B cells were exposed to increasing Cr(VI) doses, they underwent cell cycle arrest, apoptosis and necrosis. Upon chronic exposure to a mildly cytotoxic Cr(VI) dose, we were able to induce their morphological transformation. The results suggest a partial loss of contact inhibition as well as an increased resistance to Cr(VI)-induced apoptosis and they also show that Cr(VI) interferes with cell adhesion to the substratum and with cell-to-cell adhesion. It is well established that the induction of lung cancer by Cr(VI) requires chronic inhalation [1] and, to the best of our knowledge, this is the first published report on the effects of long-term chronic Cr(VI) exposures using nontumorigenic human lung epithelial cells. Moreover, it is the first direct report on Cr(VI) interference with cell adhesion to the substratum and with cell–cell interactions.

## MATERIALS AND METHODS

### Reagents

Potassium dichromate (≥99%) was obtained from Panreac Química S.A.U. (Barcelona, Spain). 1,5-Diphenylcarbazide (Sigma-Aldrich, Fluka; ≥97%), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, Sigma; ≥97.5%), gelatin type B (Sigma; 2% solution, from bovine skin) and most general reagents were purchased from Sigma–Aldrich (Madrid, Spain). Bronchial Epithelium Growth Medium (BEGM medium), medium supplements and the antibiotics amphotericin-B sulfate and gentamicin (Lonza, Clonetics®) were obtained as a kit from Lonza (Barcelona, Spain). The Vybrant® Apoptosis Assay Kit #5 (Invitrogen, Molecular Probes™), containing the fluorescent dyes Hoechst 33342 and propidium iodide, were obtained from Invitrogen (Paisley, UK).

### Cell Culture

BEAS-2B cells, obtained from the European Collection of Animal Cell Cultures (Salisbury, UK; ECCAC no. 95102433), were grown in serum-free BEGM medium supplemented with bovine insulin (5 mg/L), bovine pituitary extract, epinephrine (0.5 mg/L), human epidermal growth factor, hydrocortisone (0.5 mg/L), retinoic acid (0.1 μg/L), triiodothyronine (6.5 μg/L), transferrin (10 mg/L) and the antibiotics amphotericin-B sulfate and gentamicin. Cultures were established in tissue culture-grade flasks or multiwall plates pre-coated with gelatin (type B) and maintained at 37°C in a humidified atmosphere of 95% air/5% $CO_2$. Unless otherwise stated, cells were seeded at a density of 4000 cells/cm$^2$ (using a 20 000 cells/mL cell suspension) and were routinely subcultured at 70–80% confluence. For nonexposed cultures and for those exposed to 0.1 μM Cr(VI), this degree of confluence was normally reached 5 d after seeding. For cultures exposed to 1 and 2 μM Cr(VI), it was normally reached 7 and 9 d after seeding, respectively. Under these conditions, cells never reached the stationary phase of growth.

### Hexavalent Chromium Treatment

Appropriate amounts of potassium dichromate ($K_2Cr_2O_7$) were dissolved in ultra-pure water and the resulting solutions were sterilized through a 0.2 μm filter before use. Cells were treated for the specified times with a final concentration of 0.1–10 μM Cr(VI) in complete medium. Twenty-times concentrated Cr(VI) solutions were used in all experiments. The final media volumes were 200 μL per well for 96-well plates, 380 μL per well for 24-well plates and 5 mL for 25 cm$^2$ flasks. In experiments involving acute exposures (24, 48, and 72 h), that is, in the viability assays and in the determination of the percentage of apoptotic and necrotic cells in culture, cells were always incubated for 24 h prior to potassium dichromate exposure, to ensure proper attachment to the substratum. On the contrary, chronic exposure to Cr(VI) (at a final concentration of 2 μM or less) required that Cr(VI) be added immediately after plating. In all cases, control cultures, established and processed in parallel, received the same amount of the addition vehicle (water) as the treated cultures did.

### Cell Viability Assay

Cell viability was determined in terms of dehydrogenase activity using the MTT colorimetric assay [41]. In this assay, the seeding density had to be increased to ca. 13 300 cells/cm$^2$, in order to obtain higher absorbance values. Briefly, cultures in 96-well plates (4000 cells per 0.3 cm$^2$ well) were treated for different time periods (24–144 h) with 10 μL of 20× concentrated Cr(VI) solutions, to a final concentration of 0.1–25 μM Cr(VI). Eight replicate wells were used for each condition. At the end of the incubations, the medium was removed and 50 μL of a 0.5 mg/mL MTT solution was added. After a 3 h incubation, the MTT solution was aspirated and

the insoluble formazan crystals that formed were dissolved in 50 μL of a 0.1 M HCl solution in isopropanol. Absorbance values at 570 and 690 nm were then measured. MTT absorbance refers to the absorbance at 570 nm minus the absorbance at 690 nm.

### Determination of the Percentage of Normal, Apoptotic, and Necrotic Cells in Culture

The percentage of normal, apoptotic and necrotic cells in cultures treated with 0, 1, 2, and 4 μM Cr(VI) for 24, 48, and 72 h was determined by fluorescence microscopy [42] using the Vybrant® Apoptosis Assay Kit #5, which contained the fluorescent dyes Hoechst 33342 and propidium iodide. The experiment was carried out following the manufacturer's instructions, with some modifications. Briefly, staining was carried out in situ using cultures established in 24-well plates (1.9 $cm^2$ per well) containing coverslips pre-coated with gelatin. Cultures were treated with 19 μL of 20× concentrated Cr(VI) solutions to the desired final concentrations. Triplicate cultures were used for each condition. After treatment, coverslips were transferred to new 24-well plates containing the dyes, where cultures were incubated on ice for half an hour. At the end of this incubation, cultures were viewed under a fluorescence microscope (40× magnification) and the percentages of normal, apoptotic and necrotic cells within a minimum of three microscope fields of view were determined. The values obtained for the different fields were then averaged.

### Atomic Force Microscopy

Cultures were established in 24-well plates containing coverslips pre-coated with gelatin and treated with 0 or 2 μM Cr(VI) (0 and 19 μL of a 40 μM Cr(VI) solution, respectively). Two cultures were established for each condition. Cultures were visualized just before confluence. Prior to visualization, the biological material was fixed by immersion of the coverslips in a solution of 0.1% glutaraldehyde for 5 min. After fixation, the coverslips were washed with phosphate-buffered saline and air dried. The samples were imaged by a Nanoscope III Multimode AFM (Digital Instrumentals, Inc., Santa Barbara, CA), operating in tapping mode in air, at a scan rate of 1–3 Hz. The Atomic Force Microscopy (AFM) probe was a 125 mm-long monocrystalline silicon cantilever with integrated conical shaped Si tips (Nanosensors, Wetzlar, Germany) with an average resonance frequency $f_o = 330$ kHz and a spring constant $K = 50$ N/m. The cantilever was rectangular and the tip radius given by the supplier was 10 nm, a cone angle of 35° and high aspect ratio. In general, the images were obtained at room temperature ($23 \pm 2$°C) and the relative humidity (RH) was typically lower than 40%.

### Determination of Cr(VI) Levels in the Growth Medium

Cr(VI) levels in the growth medium were estimated colorimetrically following reaction with 1,5-diphenylcarbazide. The reaction yields a red-violet product that can be quantified at 540 nm, as described in the Test Method 7196A (Chromium. Hexavalent (Colorimetric)) of the Environmental Protection Agency (http://www.epa.gov/epawaste/hazard/testmethods/SW846/pdfs/7196a.pdf; accessed March 18 2009).

### Determination of Doubling Times

Doubling times for control cultures and for cultures chronically exposed to 0.1, 1, and 2 μM Cr(VI) were determined from the corresponding growth curves (plots of number of cells in culture against time). For these determinations, cultures ($100 \times 10^3$ cells) were established in 25 $cm^2$ tissue culture flasks (5 mL final volume; 50 μL of a 20× concentrated Cr(VI) solution) and the increase in total cell number over time was followed by direct microscope counting across a specified field (defined by a ocular square). A minimum of ten fields, chosen at random, was used in each determination. Cell countings were performed twice daily. Doubling times after Cr(VI) withdrawal were also determined for cultures that had been exposed to 1 and 2 μM Cr(VI) for multiple rounds of cell divisions.

### Statistical Analysis

The statistical significance of the differences from the control was assessed using one way ANOVA followed by Dunnett's post-test.

## RESULTS

### Establishment of Adequate Conditions for Chronic Cr(VI) Exposure

In order to establish adequate conditions for chronic Cr(VI) exposure, cultures of BEAS-2B cells were treated for 24, 48, and 72 h with Cr(VI) doses ranging from 0.1 to 25 μM. Cr(VI) was added to the cultures 24 h after seeding, to allow for proper attachment to the substratum. In fact, cells did not adhere to the substratum when Cr(VI) at a final concentration of 4 μM and higher was added immediately after seeding. Once adhesion had taken place, detachment from the substratum seemed to be confined to dead cells. In this context, it must be mentioned that, throughout this study, Cr(VI)-treated cells were consistently less difficult to trypsinize than control cells.

Cell viability was evaluated in terms of dehydrogenase activity, using the MTT assay, and expressed as a percentage of the control value (relative viability levels; Fig. 1). Cr(VI) doses higher than 4 μM were also tested (up to 25 μM) and produced considerable, time- and dose-dependent decreases in cell viability (results not shown). Rather unexpected changes in

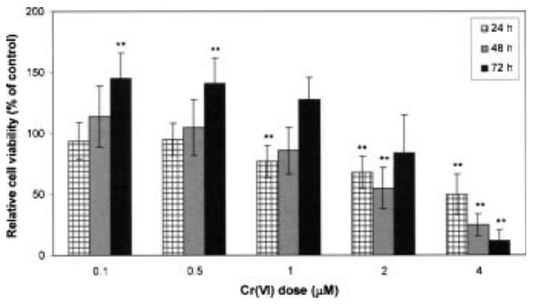


Figure 1. Viability of cultures of BEAS-2B cells after exposure to different Cr(VI) insults. For each culture, cell viability was determined in terms of dehydrogenase activity, using the MTT assay. Values are expressed as percentage of the corresponding control value, obtained with control cultures established and processed in parallel. Data represent the mean ± standard deviation (SD) of seven independent experiments. $^{**}P < 0.01$, when compared to control value, with repeated measures ANOVA followed by Dunnett's multiple comparison test.

viability were observed upon incubation with the lower Cr(VI) doses (0.1, 0.5, 1, and 2 μM Cr(VI)). After an initial decrease, viability values increased with the incubation time and, except for the 2 μM dose, eventually became higher than those of the control cultures.

Next, the effects of the 1, 2, and 4 μM Cr(VI) treatments on the percentage of dead cells in culture were determined by fluorescence microscopy. Treating BEAS-2B cells with 4 μM Cr(VI) elicited a very significant, time-dependent increase in the percentage of dead cells in culture (both apoptotic and necrotic; Table 1). Treatment with 2 μM Cr(VI) also resulted in an increase in the percentage of apoptotic cells in culture, but much less pronounced than that induced by 4 μM Cr(VI). Necrosis levels remained similar to those of the controls. Very importantly, the percentage of apoptotic cells in cultures treated with 2 μM Cr(VI) not only did not increase upon extending the incubation period, but actually decreased. A similar finding was reported by Singh and co-workers for normal human bronchial epithelial (NHBE) cells [43].

Altogether, these initial results evidenced a clear threshold of toxicity between 2 and 4 μM, and the use of Cr(VI) doses of 4 μM and higher was, therefore, discontinued.

### Effects of Cr(VI) Exposure on Contact Inhibition

The results obtained in a new set of viability assays designed to gain further insight into the reasons why some Cr(VI)-exposed cultures exhibited viability values higher than those of the controls are summarized in Figure 2. As can be seen, cell growth between 72 and 96 h post-Cr(VI) addition was very small for both control and treated cultures. This was likely due to contact inhibition, as direct microscopic observation revealed that the degree of confluence at 72 h was already very high. This growth arrest was significantly less pronounced for Cr(VI)-treated cultures than for the control cultures, which could be explained in terms of a partial loss of contact inhibition. After ca. 24 h at confluence, both control and Cr(VI)-treated cultures were able to resume growth, but viability soon leveled off, probably due to nutrient exhaustion.

### Atomic Force Microscopy

AFM images of collagen-coated culture surfaces treated with 2 μM Cr(VI) clearly show the destruction of the collagen network (the main constituent of the extracellular matrix (ECM)) (Fig. 3A and B), as well as alterations in the cell membrane surface upon treatment of confluent cultures of BEAS-2B cells with this Cr(VI) dose (Fig. 3C–E).

### Changes in Morphology and Pattern of Growth Induced by Chronic Cr(VI) Exposure

Four Cr(VI) doses, either subcytotoxic or mildly cytotoxic (0.1, 0.5, 1, and 2 μM), were chosen for chronic exposure (over 15 passages). The experimental conditions chosen for these chronic exposures

Table 1. Percentage of Apoptotic and Necrotic Cells in Cultures of BEAS-2B Cells Exposed to Different Cr(VI) Insults

| | Percentage of dead cells in culture[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Apoptotic cells | | | | Necrotic cells | | | |
| Exposure time (h) | 0 μM | 1 μM | 2 μM | 4 μM | 0 μM | 1 μM | 2 μM | 4 μM |
| 24 | 2 ± 1 | 3 ± 3 | 9 ± 6 | 11 ± 6* | 2 ± 2 | 2 ± 2 | 3 ± 1 | 5 ± 4 |
| 48 | 2 ± 1 | 2 ± 3 | 7 ± 6 | 18 ± 5** | 1 ± 0 | 1 ± 1 | 4 ± 1 | 13 ± 12* |
| 72 | 1 ± 1 | 1 ± 1 | 6 ± 5 | 24 ± 18** | 1 ± 0 | 1 ± 1 | 3 ± 2 | 28 ± 22** |

Data represent the mean ± SD of at least three independent experiments.
[a]The percentage of apoptotic and necrotic cells in culture was determined by fluorescence microscopy using the fluorescent dyes Hoescht 33342 and propidium iodide.
$^{*}P < 0.05$, when compared to control value, with ANOVA followed by Dunnett's multiple comparison test.
$^{**}P < 0.01$, when compared to control value, with ANOVA followed by Dunnett's multiple comparison test.

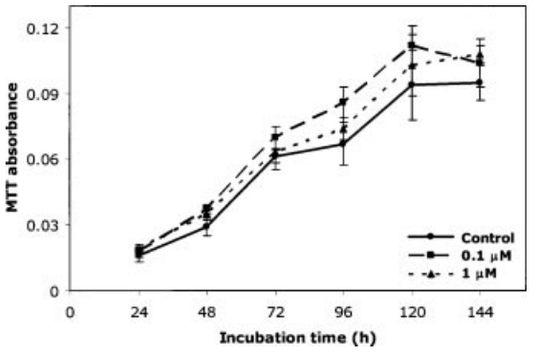


Figure 2. Cell viability, expressed in terms of dehydrogenase activity, of cultures of BEAS-2B cells exposed to different Cr(VI) insults. Values represent means of either two (cultures exposed to 1 μM Cr(VI)) or three (control cultures and cultures exposed to 0.1 μM Cr(VI)) independent experiments. Bars represent the corresponding SD. Due to the very high seeding density used in this assay, the degree of confluence at 72 h was very high. This had severe effects on the growth rate, due to contact inhibition. Growth arrest was particularly severe for control cultures, for which practically no growth was observed between 72 and 96 h. The arrest was significantly less pronounced for Cr(VI)-treated cultures, which could be accounted for by a partial loss of contact inhibition. After ca. 24 h of growth arrest, cells from all the three distinct cultures were able to resume growth. At ca. 120 h, cultures reached the plateau phase of growth.

(namely, the seeding density) ensured that cultures never reached the plateau phase of growth. Chronic exposure involved adding Cr(VI) immediately after passaging and, although in the presence of these low Cr(VI) doses cells did attach to the substratum, it was noticeable, by direct microscope observation, that cultures exposed to 1 and 2 μM Cr(VI) took longer to resume proliferation (i.e., their ''lag phases'' increased). Results obtained in two preliminary experiments (results not shown) using 2 μM Cr(VI) and where the total number of cells in culture and Cr(VI) levels in the growth medium were monitorized simultaneously showed that the onset of proliferation was delayed until these levels lowered below ca. 1 μM (the sensitivity of the method used did not allow for an exact determination of the threshold value).

In terms of morphology and growth pattern, no significant changes were observed between cultures chronically exposed to 0.1 or 0.5 μM Cr(VI) and control cultures. Throughout the exposure, confluent cultures were, in all three cases, very homogeneous, with a strong predominance of dense, cobblestone-like monolayers of cells displaying morphological characteristics typical of NHBE cells (Fig. 4). On the contrary, cultures chronically exposed to 1 or 2 μM Cr(VI) exhibited changes in morphology and pattern of growth (Fig. 4). Upon chronic treatment, cultures became heterogeneous, showing areas of ''cobblestone'' growth interspersed with areas of cells with an altered morphology and/or pattern of growth. In some areas, cells, while retaining the diamond-shape of NHBE cells, grew

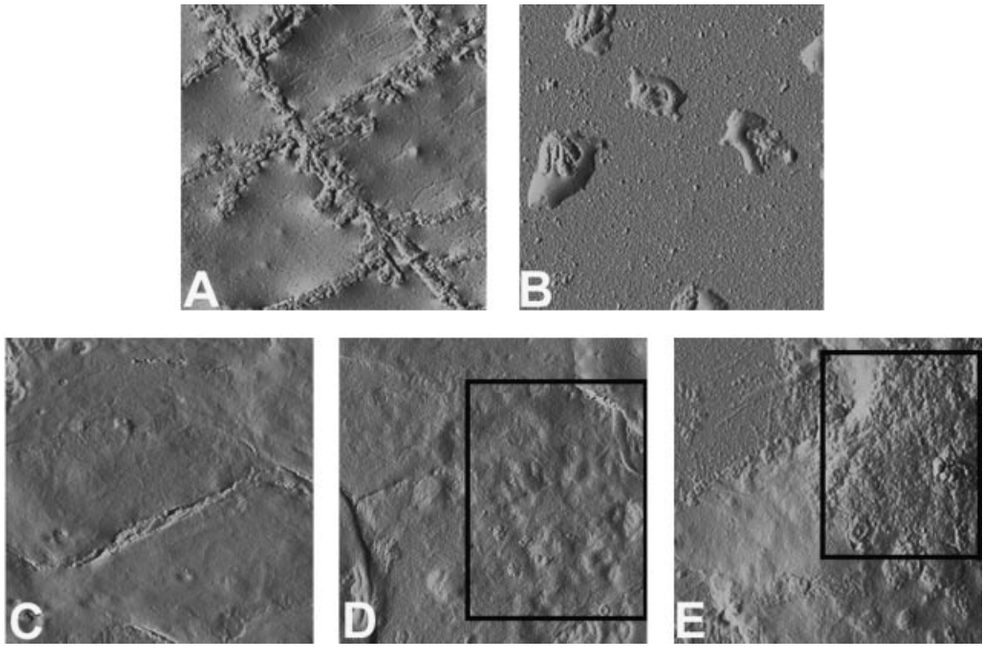


Figure 3. (A) AFM image of a collagen-coated culture surface, clearly showing the collagen network; (B) AFM image of a collagen-coated culture surface after treatment with 2 μM Cr(VI). (C) AFM image of a confluent monolayer of control bronchial epithelial cells (BEAS-2B). (D and E) AFM images of confluent monolayers of bronchial epithelial cells (BEAS-2B) after treatment with 2 μM Cr(VI); the boxes highlight zones of the cellular membrane showing morphological alterations. All AFM images are representations of AFM amplitude data scanned within a 15 × 15 μm square using the tapping mode in air.

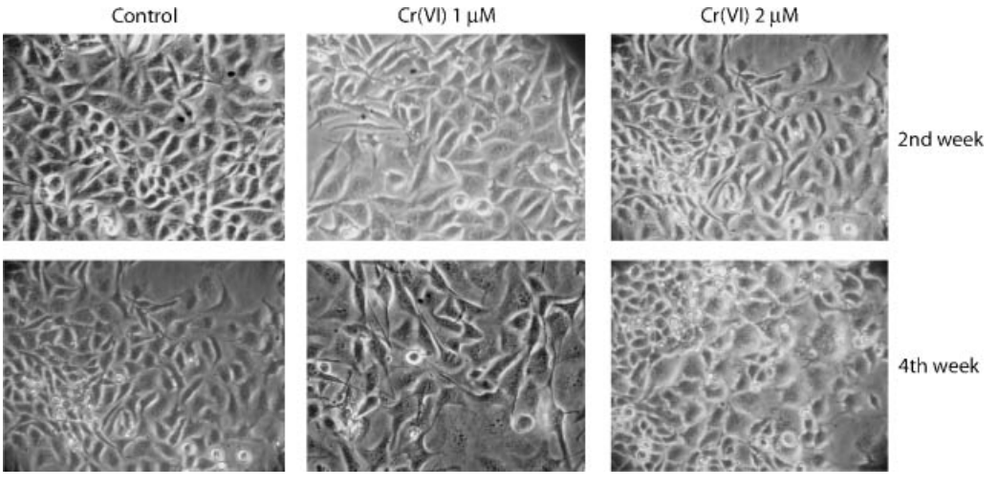


Figure 4. Effects of Cr(VI) treatment on the morphology and pattern of growth of BEAS-2B cultures (phase contrast micrographs; 20×). At confluence, control BEAS-2B cells always gave rise to typical epithelial monolayers. BEAS-2B cells exposed to 1 or 2 μM Cr(VI) exhibited morphologies and patterns of growth distinctively different from those of the control cultures. These differences were already detectable after 2 wk of Cr(VI) treatment. Shown here are areas of the Cr(VI)-treated cultures where even those cells that retained the diamond-shape of NHBE cells grew with no formal pattern. Moreover, there was a marked increase in the number of giant cells.

with no formal pattern. Other areas had a completely disorganized appearance and contained cells that exhibited a variety of bizarre shapes. A significant increase in the incidence of irregular networks of criss-crossing, spindle-shaped cells and in the number of round and refractile cells was also observed. Moreover, there was a marked increase in the number of multinucleated, greatly enlarged cells with increased granularity. Interestingly, in cultures chronically exposed to 2 μM Cr(VI), there were also areas where quadrangular-shaped cells formed a honeycomb-like arrangement where cells did not pack as densely as in the ''cobblestone'' monolayers.

The effects of chronic Cr(VI) exposure on the doubling time were also investigated (Table 2). Whereas exposure to 0.1 or 0.5 μM Cr(VI) had no effect in the growth rate of the BEAS-2B cultures, the doubling times of cultures chronically exposed to 1 μM (28 h) and 2 μM Cr(VI) (36 h) were higher than those of the control (22 h). In spite of these delays in cell cycle, cultures remained viable. In each experiment, cultures were followed for a minimum of three passages, and no significant changes in doubling time were observed over time for the same type of culture.

## DISCUSSION

Despite extensive studies, the mechanisms underlying Cr(VI)-induced lung cancer remain largely unknown, partly due to the rather limited options for suitable experimental models. Animal models are expensive and their tracheobronchial tree is not readily accessible. The time nontransformed bronchial cell lines can be maintained in culture is relatively short, precluding mechanistic investigations [44,45]. Hence, the majority of in vitro studies of Cr(VI)-induced bronchial carcinogenesis were carried out using immortal cell lines, including fully neoplastic cells, whose properties are likely to affect the carcinogenic process being modeled [46,47]. Also, although Cr(VI) predominantly induces squamous lung cancer [48], there are few reports of studies using nontumorigenic human bronchial epithelial cells [38,43,49–61], notwith-

Table 2. Effects of Different Cr(VI) Doses on the Doubling Times of BEAS-2B Cells

| Doubling times (h) for cultures treated with different Cr(VI) concentrations[a] | | | | |
|---|---|---|---|---|
| 0 μM | 0.1 μM | 0.5 μM | 1.0 μM | 2.0 μM |
| 22 ± 3 | 21 ± 1 | 24 ± 1 | 28 ± 3* | 36 ± 8** |

[a]Values are from at least four independent experiments and represent means ± SD. In each independent experiment, cultures were normally followed for at least three passages.
*$P < 0.05$, when compared to control value, with ANOVA followed by Dunnett's multiple comparison test.
**$P < 0.01$, when compared to control value, with ANOVA followed by Dunnett's multiple comparison test.

standing the fact that different cell types may differ in terms of uptake, metabolism and responses to Cr(VI).

In this study, we used the immortalized BEAS-2B cell line, derived from normal human bronchial epithelium through infection with an adenovirus 12-simian virus 40 (SV40) hybrid virus preparation [45]. These cells produce the SV40 T antigen, which binds the tumor suppressor proteins p53 and Rb, predisposing cells to transformation [62–65]. Although not normal, these cells retain many differentiated characteristics of NHBE cells and are considered nontumorigenic [45,66–68]. In particular, BEAS-2B cells respond to treatment with serum or transforming growth factor $\beta_1$ by ceasing cell division and undergoing terminal squamous differentiation [66], they are able to sustain the expression of exogenous wild-type (WT) p53 [67] and they do not form tumors in immunosuppressed mice [45,66–68], all hallmarks of NHBE cells. Also, studies by Gerwin et al. [67] have established that, in these cells, the presence of the T antigen does not result in a completely loss of WT-p53 function.

It has been shown that high levels of Cr-DNA binding lead to inhibition of polymerase activity [27], triggering apoptosis, which prevents carcinogenesis. Lower levels of Cr-DNA binding increased polymerase activity and processivity, with a concomitant decrease in the fidelity of DNA replication [27]. As there is also evidence that the mutagenic and transforming actions of Cr(VI) occur at doses that produce some cell death [19,20], Cr(VI) doses of low cytotoxicity should be favored in this type of study. Finally, considering that the development of lung cancer by Cr(VI) requires chronic inhalation, long-term chronic exposures, as opposed to the short-term assays that predominate in the literature, should be considered.

The aim of the initial experiments was to establish exposure conditions closer to the in vivo situation. The first of several observations of Cr(VI) interference with cell attachment to the substratum was made at this stage: cells exposed to Cr(VI) doses of 4 μM and higher immediately after seeding did not adhere to the substratum. This could also reflect cell death, if BEAS-2B cells still in suspension were significantly more sensitive to Cr(VI) than their attached counterparts. Nonetheless, Cr(VI)-treated cells were consistently less difficult to trypsinize than control cells, revealing a reduced adhesiveness to the substratum. Since adhesion and subsequent spreading are critical for proliferation of normal adherent cells [69], permanent interference with these processes due to chronic Cr(VI) exposure may give rise to a population of cells able to proliferate in anchorage-free conditions, an important step in the establishment of metastases. After adhesion had taken place, Cr(VI) addition did not revert it.

These initial experiments suggested other Cr(VI) effects that may be important in the context of its carcinogenicity, namely resistance to Cr(VI)-induced apoptosis and a lower sensitivity to contact inhibition. When BEAS-2B cells were exposed for 24 h to 0.1, 0.5, 1, and 2 μM Cr(VI), there was a dose-dependent decrease in viability. Rather unexpectedly, these decreases were reverted upon extending the incubation time and, except for the 2 μM dose, viability values eventually became higher than those of the control cultures. This reversal suggests an increased tolerance to Cr(VI), but there are some confounding factors, such as the decrease in Cr(VI) levels in the growth medium that was observed upon time in culture, due to a fast inflow through the phosphate/sulfate carrier [36,38,70–73]. Moreover, the degree of confluence at 48 h post-Cr(VI) addition was already considerable for control cultures, inhibiting cell growth due to contact inhibition. Cultures treated with 1 and 2 μM Cr(VI) exhibited lower degrees of confluence, due to more pronounced initial decreases in cell viability (Fig. 1). Therefore, the growth rate at 48–72 h post-Cr(VI) addition may have been higher for these exposed cultures than for control cultures. Notwithstanding, the results obtained in the determination of the percentage of dead cells in culture, which, due to a lower seeding density, was carried out under conditions of a much lower Cr(VI) influx and where cultures were never allowed to reach the plateau phase of growth, seem to confirm the acquisition of an increased tolerance to Cr(VI) shortly after the initial insult. This ability to escape apoptosis may allow cells to progress to a tumorigenic phenotype. As to the viability values higher than those of the control cultures, they were only observed when the degree of confluence was already considerable for all cultures, suggesting that Cr(VI)-treated cells were less sensitive to contact inhibition. However, more conclusive experimental data is needed. The decreases in cell viability observed upon 24 and 48 h treatments with 1 μM Cr(VI) were not due to cell death, but possibly to a delay in the onset of proliferation as a consequence of Cr(VI) interference with cell adhesion to the substratum and/or to a transient cell cycle checkpoint arrest to repair DNA damage.

AFM was used to gain a better insight into the potential interference of Cr(VI) with cell adhesion. Treating BEAS-2B cells with 2 μM Cr(VI) induced significant alterations in both the ECM collagen network and the cell membrane surface that can have affected the cells' ability to establish interactions with each other and/or with the substratum. As it is now accepted that changes in the ECM and loss or modifications in cell–cell and cell–ECM interactions are involved in transformation [74,75], it is tempting to speculate that these extracellular events may indeed contribute to Cr(VI)-induced carcinogenesis.

Four subcytotoxic or mildly cytotoxic Cr(VI) doses were chosen for chronic exposure. Continuous passaging in the presence of 0.1 or 0.5 μM Cr(VI) had no effect on the morphology and pattern of growth of the BEAS-2B cells. On the contrary, incubation with 1 and 2 μM Cr(VI) had profound effects. Cultures became very heterogeneous and exhibited several altered phenotypes: there were patches where cells grew with no formal pattern (likely a consequence of altered cell–cell contacts), criss-crossing of cells became a common feature, and a whole spectrum of altered cell shapes could be observed. In cultures treated with 2 μM Cr(VI), some colonies exhibited distinctive spaces between the cells, reflecting a change in their capability to establish and/or maintain cell–cell contacts. Changes in cell morphology also support the hypothesis that Cr(VI) interferes with the interactions of cells with the substratum and/or with other cells, as these interactions are known to influence cell shape [76]. Another important observation was the accumulation of multinucleated, giant cells. This senescence-like status, known as accelerated or premature senescence, has been observed in cultures exposed to DNA-damaging agents [77–79] and in cultures of primary cells made to express oncogenic *ras* [80]. In our study, the accumulation of these cells likely paralleled the accumulation of irreparable DNA damage upon prolonged Cr(VI) exposure. This result clearly shows that, although immortalized, BEAS-2B cells are still capable of undergoing senescence in response to potentially oncogenic events. Although accelerated senescence was suggested to be a dynamic protective response to potential carcinogenic stimuli [81,82], it has also been postulated that a small minority of senescent cells may, in the course of neoplastic transformation, escape this state and grow as clones [83,84]. Moreover, as senescent cells can alter the tissue microenvironment, it was proposed that these cells can stimulate tumorigenesis [85].

No significant changes were observed on the doubling times of cultures exposed to 0.1 or 0.5 μM Cr(VI), showing that the relative viability values higher than 100% obtained upon acute exposures (Fig. 1) were not due to a growth stimulation. On the contrary, the higher doses resulted in a profound, dose-dependent effect on this parameter that could be accounted for by extended ''lag phases'' due to Cr(VI) interference with cell adhesion to the substratum and by a significant cell-cycle delay. An increased doubling time upon long-term chronic exposure to 1 μM Cr(VI) was also reported for the human cervix carcinoma cell line NHIK 3025 [86].

In conclusion, this study describes a variety of Cr(VI)-induced effects that should be important in the context of its carcinogenicity. To the best of our knowledge, this is the first report on the effects of a long-term, chronic treatment of human bronchial epithelial cells with Cr(VI). In particular, one aspect of this research that deserves further investigation is the potential interference of this carcinogen with cell–cell interactions and with cell adhesion to the substratum, possibly through interaction with membrane proteins and/or by altering the adhesion properties of the substratum. In fact, viewing cancer merely as the result of deregulated signaling pathways is no longer acceptable and increasing attention has been devoted to the role that the ECM plays in the process of carcinogenesis.

The study here discussed is being extended by our group and has already included the generation and initial characterization of cell strains obtained from cultures of BEAS-2B cells that were chronically exposed to 1 μM Cr(VI) [87]. Importantly, when cells from one of these strains were inoculated into nude mice, they showed the ability to induce tumors.

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