Mechanism of Apoptosis and Determination of Cellular Fate in Chromium(VI)-exposed Populations of Telomerase-immortalized Human Fibroblasts

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Abstract

The cellular responses to carcinogen exposure influence cellular fate, which in turn modulates the neoplastic response. Certain hexavalent chromium [Cr(VI)] compounds are implicated as occupational respiratory carcinogens at doses that are both genotoxic and cytotoxic. We examined the mechanism of Cr(VI)-induced apoptosis in normal human fibroblasts (BJ) immortalized by human telomerase gene transfection (BJ-hTERT), and we assessed the spectrum of cumulative cellular fates [i.e., regaining of replicative potential; (b) terminal growth arrest; or (c) apoptosis] for a narrow range of increasingly genotoxic doses of Cr(VI). Exposure of BJ-hTERT cells to Cr(VI) resulted in a dose-dependent increase in apoptosis that involved mitochondrial disruption as evidenced by mitochondrial membrane depolarization and cytochrome c release. The initial response to Cr(VI) exposure was inhibition of cell cycle progression. At the lowest dose tested (1 μM; 32% clonogenic survival), the cell cycle inhibition led to terminal growth arrest but no apoptosis. The fraction of terminally growth arrested cells increased as the dose was increased to 3 μM but then decreased at 4, 5, and 6 μM as apoptosis became the predominant cell fate. Our results suggest that cell populations exposed to Cr(VI) have a different spectrum of responses, depending on the extent of DNA damage, and that the regaining of replicative potential after relatively higher genotoxic exposures may be attributable to either escape from, or resistance to, terminal growth arrest or apoptosis.

Introduction

Cellular responses to genotoxic stress are believed to provide a protective effect against tumor development by preventing the outgrowth of cells with potential oncogenic alterations. DNA damage may cause cell cycle delay, presumably to provide an opportunity for a cell to repair the lesions before replication (1). When damage is irreparable, cells must be removed from the proliferating population to control propagation of damaged DNA. The predominant cellular fates, in response to irreparable DNA damage, are terminal growth arrest and apoptosis (2). The responses to genotoxic stress that lead to these cellular fates are mediated largely by p53 (3). The final cellular outcome of p53 activation depends on many factors and is mediated, in part, through the transcriptional activation of downstream effector genes that are involved in cell cycle arrest, DNA repair, and apoptosis (4, 5). In the case of a genotoxic agent such as Cr(VI), the magnitude of exposure determines the extent of DNA damage. It is generally assumed that the extent of damage may be a major determinant in the cellular response, but this hypothesis has not been critically tested, other than gross evaluations of apoptosis versus necrosis. In this study, we evaluated the cellular responses and the cumulative population outcomes of BJ-hTERT fibroblasts exposed to a narrow range of increasingly genotoxic doses of Cr(VI).

Inhaled particulate forms of Cr(VI) are strongly associated with lung toxicity and increased incidence of lung cancer (6, 7). These adverse health effects are usually associated with occupational exposure, but the generation of chromium waste by chromate industries has also raised concerns about Cr(VI) as a potential environmental hazard (8–11). The main targets for Cr(VI) toxicity are lung epithelial cells and fibroblasts exposed to high concentrations of soluble Cr(VI) in the immediate microenvironment of inhaled particles (12, 13). Thus, soluble sodium chromate (Na₂CrO₄) can be used to study the genotoxic and cytotoxic effects of Cr(VI) in cell culture. Cr(VI) ions enter cells through an anion transporter, where they undergo metabolic reduction to reactive genotoxic species (14–16). These products, as well as the oxidative stress generated by the reduction process, lead to diverse genotoxic and cytotoxic effects including structural and functional DNA damage. The spectrum of structural DNA damage includes DNA adducts (17, 18), DNA-DNA cross-
links (19–21), DNA-protein cross-links (18), and chromosomal damage (22, 23). This structural damage may lead to functional damage in the form of DNA and RNA polymerase arrest (24, 25), mutagenesis (26, 27), and/or altered gene expression (28, 29). We have shown previously that treatment of HLFs and human small airway epithelial cells with the concentrations of Na₂CrO₄ used in this study causes a dose-dependent increase in Cr-DNA adduct formation (17, 18), functional DNA damage (24–29), and apoptotic cell death (12, 30, 31). Similar to most reactive chemical species, the products of reductive metabolism of Cr(VI) can react with and damage other biomolecules and organelles besides DNA, and these reactions may also contribute to, or occur in addition to, genotoxic damage. We have also shown that Cr(VI) exposure results in activation of p53 (12), and that Cr(VI)-induced apoptosis in HLFs is p53 dependent (32). However, those studies did not compare the different possible cellular outcomes of Cr(VI) exposure over time and did not fully explore the mechanism or dose dependence of Cr(VI)-induced apoptosis.

The goal of the present study was to: (a) more fully explore the mechanism of Cr(VI)-induced apoptosis; and (b) characterize the dose-dependent cellular responses to this genotoxin in a population-based cumulative index. To accomplish this, we had to overcome a major limitation of normal diploid human fibroblasts in culture, which change their responses to genotoxicity as the population approaches senescence (33, 34). Therefore, we used human fibroblasts (BJ) immortalized by telomerase transfection (BJ-hTERT; Geron Corp., Menlo Park, CA) for these experiments. These telomerase-expressing cells have an extended life span but have not been transformed with viral oncoproteins, which extend cell life span through mechanisms that also reduce cell cycle checkpoint control and increase genomic instability (35, 36). BJ-hTERT cells display anchorage-dependent growth, retain chromosomal integrity, are nontumorigenic, maintain an intact p53/Rb axis, and can rejoin DNA double-strand breaks normally (37, 38). However, they are not limited by senescence in long-term cell culture experiments (38). Thus, BJ-hTERT cells provide an excellent model for studying Cr(VI) toxicity because they are immortal but maintain a normal response to genotoxic stress.

Many of the standard techniques used to measure apoptosis and growth arrest are limited because they quantitate an aspect of these fates at a particular instant in time. Here, we provide a dynamic population study of Cr(VI)-exposed cells by determining clonogenic potential, tracking cell growth, and determining cellular fates, over time. We examined the cellular fates at increasingly genotoxic doses and expressed them as the cumulative percentages of CGA index. The CGA index shifted from clonogenic survival to terminal growth arrest and then to apoptosis as the degree of genotoxicity increased. Even at the lowest dose tested, a period of population-wide, transient cell cycle arrest was followed by replicative recovery and outgrowth of only a subset of cells within the population. The balance of cells converted from transient to terminal growth arrest. At the higher doses, our results suggest that the initial population-wide cell cycle arrest in response to Cr(VI) exposure is followed by two possible dose-related terminal cellular fates: terminal growth arrest or apoptosis. The third fate, regaining replicative potential, would appear to require escape from or resistance to either or both of these terminal fates.

Results

We used human foreskin fibroblasts immortalized by human telomerase gene transfection (BJ-hTERT; Geron Corp.) for these experiments because of their stable phenotype in cell culture. We had found previously that normal diploid human lung fibroblasts lost the ability to undergo genotoxin-induced apoptosis with progressive cell culture passage (12). Thus, it was crucial to determine whether the cells used in these experiments had an altered susceptibility to genotoxin-induced apoptosis with cumulative cell divisions. We compared BJ-hTERT cells to the parental BJ cell line for susceptibility to Cr(VI)-induced apoptosis at increasing cell culture passage numbers using the phosphatidylserine translocation assay (Fig. 1). Early passage BJ [passage 7 (p7)] exhibited the highest susceptibility to Cr(VI)-induced apoptosis, but this “apoptosis-permissive” state was markedly decreased by p12–p17 and was nearly absent by p21–p26 (~90 population doublings). In contrast, the incidence of Cr(VI)-induced apoptosis in BJ-hTERT cells exhibited no significant change over 19 subsequent cell culture passages (p127–p146); thus, the data from nine separate passages within p127–p146 were pooled and shown as a single bar with minimal SE. The reduced susceptibility to Cr(VI)-induced apoptosis in BJ fibroblasts corresponded with decreased cell growth, increased cell size, and increased ratio of cytoplasm: nucleus (data not shown), which are typical characteristics of cells progressing toward senescence (39).

The apoptotic process has been demonstrated to be linked to mitochondrial integrity (reviewed in Ref. 40). To
determine whether Cr(VI)-induced apoptosis in BJ-hTERT involves mitochondrial disruption, we examined the depolarization of the mitochondrial membrane by measuring the fluorescence emission shift of the cationic JC-9 dye (Molecular Probes, Eugene, OR). JC-9 exists in monomeric form, emitting at 525 nm (green fluorescence) after excitation at 490 nm. At relatively high mitochondrial membrane potential (ΔΨm), JC-9 forms aggregates within mitochondria with a fluorescence emission at 590 nm (orange fluorescence). Mitochondrial membrane depolarization is indicated by an increase in green fluorescence. H2O2 (0.5 mM for 15 min) and valinomycin (5 μM for 24 h) caused an increase in depolarization to 99 and 65% of total cells, respectively (data not shown). A 24-h exposure to different concentrations of Na2CrO4 enhanced mitochondrial membrane depolarization in a dose-dependent fashion (Fig. 2). The increase in total cells with depolarized mitochondria was statistically significant at the 9 μM Na2CrO4 dose (P < 0.05), at 2-fold above the control.

The release of cyt c from the mitochondria to the cytosol is necessary for Cr(VI)-induced apoptosis (41, 42) and may be directly related to mitochondrial depolarization (40). We examined cyt c release immunohistochemically in BJ-hTERT cells exposed to 0, 6, or 9 μM Na2CrO4 for 24 h using confocal laser scanning microscopy with a cyt c-specific antibody and a fluorescent secondary antibody (Fig. 3). Control cells exhibited a punctate staining pattern of intracellular cyt c indicative of mitochondrial localization (Fig. 3A). At the 6 μM dose, we observed a more diffuse intracellular staining pattern that suggests that cyt c had been released from some mitochondria to the cytosol (no cyt c is observed in the nucleus; Fig. 3B). At the 9 μM dose, we observed a marked diffusion of cyt c throughout the cytosol (Fig. 3C). Cells treated with 0.8 mM H2O2 for 15 min (positive control) had a similar morphology and cyt c staining pattern as the cells treated with 9 μM Na2CrO4 for 24 h (Fig. 3D).
Once released from the mitochondria, cyt c forms a complex with APAF-1 and procaspase 9, thereby triggering caspase 9 activation and initiating the caspase cascade (41–43). Caspase 3 is a key regulatory and effector caspase in the apoptotic pathway (44, 45). Caspase 3 activation was evaluated in BJ-hTERT cells exposed to Na$_2$CrO$_4$ for 24 h (Fig. 4). We observed a dose-dependent increase in caspase 3 activity as the Na$_2$CrO$_4$ concentration increased from 3 to 9 μM. The 3 μM dose did not cause a detectable increase in caspase 3 activity at the time point tested (24 h after a 24-h exposure); however, the 6 and 9 μM doses caused a highly significant 6- and 25-fold increase in caspase 3 activity, respectively, as compared with control (P < 0.05).

Clonogenicity is an indicator of long-term cell survival and replicative potential after exposure to a toxic agent. By determining the clonogenicity of Cr(VI)-exposed cells, we were able to examine the cumulative effect of Cr(VI) exposure on a cell population, rather than at one particular time point after exposure, as was necessary for the previous biochemical determinations. Clonogenicity was evaluated in BJ-hTERT cells exposed to different concentrations of Na$_2$CrO$_4$ for 24 h (Fig. 5). A dose-dependent decrease in clonogenicity was observed as the Na$_2$CrO$_4$ concentration increased from 0 to 5 μM. At the 6 μM dose and at doses >6 μM (data not shown), no colonies were observed.

Because the single time point determinations of Cr(VI)-induced apoptosis could not account for the marked decreases in clonogenicity, we examined BJ-hTERT cell growth over an 8-day period in populations of cells exposed to 0–6 μM Na$_2$CrO$_4$ for 24 h (Fig. 6). Na$_2$CrO$_4$ was added to samples at day 0 and was removed after 24 h. An increase in cell number was observed at all of the concentrations tested during the Cr(VI) treatment (days 0–1). The population of cells that did not receive Cr(VI) treatment (0 μM) continued to divide and exhibited an increase in cell number throughout the 8-day time course. At the lowest dose tested (1 μM), only a slight increase in cell number was observed through day 4, followed by a pronounced increase in cell number from day 4 through day 8. At the 2 and 3 μM doses, a decrease in total cells occurred through day 4, followed by a gradual increase in cell number from day 4 through day 8. At the 4 and 5 μM doses, cell loss was observed through day 4, followed by a protracted growth arrested period through day 7 for which no additional cell loss or cell growth was observed. A modest increase in cell number was observed in these populations at day 8. At the highest dose tested (6 μM), cell loss was observed through day 5, followed by a period of no change in cell number through day 8. We did not observe an increase in cell number in this population through 20 days in culture (data not shown).

To elucidate the respective outcome of cells in populations exposed to Cr(VI), we determined the cumulative cellular fate (by use of the CGA index) over the course of 2 weeks after exposure of populations of BJ-hTERT cells to different concentrations of Na$_2$CrO$_4$ for 24 h. Previous studies demonstrated that apoptosis is the predominant mode of cell loss after Cr(VI) exposure. Thus, the total percentage of apoptotic cells in a population was determined by observing the total number of cells lost over 8 days from the maximum cell number determined at day 1. The percentages of apoptotic cells and clonogenic survivors for each Cr(VI) dose were compared, and the percentage of cells that were not accounted for in these two groups was assumed to be growth arrested. The presence of growth arrested (apparently viable but nondividing) cells was confirmed by microscopic observation and deemed to be terminal when no resumption of replication could be seen for 20 days. The CGA index is expressed as a stacked bar graph indicating the percentages of clonogenic survivors, growth arrested cells, and apoptotic cells within the entire population for each Cr(VI) dose (Fig. 7). The percentage of clonogenic survivors decreased as the Cr(VI) dose increased from 0 to 6 μM. At the lowest dose
tested (1 μM), 67.9% of the cells exhibited growth arrest, but no apoptosis was detected. At the 2 μM dose, the fraction of growth arrested cells decreased to 55.9%, and apoptotic cells were clearly evident. As the Cr(VI) concentration increased from 2 to 3 μM, the apoptotic fraction did not increase any further. Instead, an incremental decrease in clonogenic survival at this dose was accompanied by an incremental increase in the growth arrested fraction (CGA index, 2.4/67.4/30.2). As the dose increased from 4 to 6 μM, the survival fraction was minimal, and a dose-dependent increase in the apoptotic fraction was accompanied by a decrease in the growth arrested fraction.

Discussion
The population-wide cell cycle arrest in response to DNA damage provides the opportunity for a cell to regulate its own proliferation, thereby avoiding the propagation of damaged DNA. Depending on the extent of the genotoxic insult, an arrested cell could either regain its replicative potential or be removed from the dividing population. In cultures of human fibroblasts, the cells that cannot overcome the genotoxic insult may be eliminated from further proliferation by undergoing either terminal growth arrest or apoptosis (46–48). We have shown previously that p53 is activated in response to Cr(VI)-induced DNA damage in normal human fibroblasts (32), and it is believed that p53 activation causes a transient arrest that may provide a chance for a cell to repair DNA damage before replication continues. p53 has also been implicated as a molecular determinant of both apoptosis and G1 terminal arrest (49), but it is not clear what factors determine the possible cellular fates of: (a) regaining of replicative potential (clonogenicity); (b) terminal growth arrest; or (c) apoptosis. One important factor may be the extent of DNA damage. Therefore, at the population level, it would be valuable to know the fate of cells at different genotoxic doses. In this study, we evaluated the cumulative cellular responses to genotoxic Cr(VI) exposure and we examined the shifts in cellular fate within populations exposed to a narrow range of increasing doses of Cr(VI).

As well as contributing to the broad understanding of cellular responses to genotoxicity, these studies have specific application to chromium carcinogenesis. Exposure to levels of particulate Cr(VI) compounds that are able to induce gene mutation and neoplastic transformation usually also induces some degree of apoptosis (6). For example, Cr(VI)-related lung cancers are often associated with respiratory toxicity involving high levels of cell death, such as perforation of the nasal septum and/or respiratory tract ulcerations (6). Cr(VI)-induced apoptosis may be a result of the DNA damage response to Cr(VI) genotoxicity, and we have shown previously that treatment of cells with Cr(VI) causes structural DNA damage in the form of adducts (17, 18), single-strand breaks (50–52), DNA cross-links (19–21), and chromosomal abnormalities (22, 23). These lesions may cause cells to undergo G1-S and S-phase cell cycle arrest, terminal growth arrest, or apoptosis, and survivors may exhibit DNA deletion mutations or altered gene expression and may progress to
neoplastic transformation (reviewed in Ref. 53). Furthermore, the products of reductive metabolism of Cr(VI) may react with other intracellular biomolecules or generate oxidative stress that may potentially contribute to the activation of pathways leading to growth arrest or apoptosis. Thus, it is important to understand the relationship between chromium exposure, apoptosis, and terminal growth arrest. We show here that Cr(VI)-exposed BJ-hTERT human fibroblasts undergo a dose-dependent increase in apoptosis (Fig. 1), and that the apoptotic pathway involves mitochondrial disruption as indicated by mitochondrial membrane depolarization (Fig. 2) and cyt c release (Fig. 3) as well as caspase activation (Fig. 4).

In culture, there is a progressive change in the phenotype of HLFs with cumulative cell divisions. One such observation in late passage HLFs is an increased resistance to apoptosis (12, 33, 34), and this is shown with BJ parental fibroblasts in Fig. 1. The altered apoptotic susceptibility in these cells may be relevant to the response to Cr(VI) exposure in vivo in individuals with long-term repetitive exposures. Lung tissue that has undergone cellular turnover in response to recurrent cytotoxic Cr(VI) exposures may present an attenuated response to subsequent exposures. Moreover, cells that survive genotoxic doses of Cr(VI) may, in turn, present an increased transmission of DNA damage. This implies that individuals with longer term occupational exposures may be more susceptible to Cr(VI)-induced lung cancers due to selection for apoptosis-resistant cells.

In contrast to the parental BJ cells, BJ-hTERT fibroblasts are sensitive to Cr(VI)-induced apoptosis, even after 146 cell culture passages, and this sensitivity does not change with subsequent cell divisions (Fig. 1). It is believed that as human cells in culture continue to divide, they undergo telomere shortening, thereby increasing the likelihood of telomere uncapping (54, 55). The presence of active telomerase in BJ-hTERT cells prevents telomere uncapping and preserves the proliferative potential of the population (56–58). Although it may be possible to induce early senescence in some cell types (59, 60), BJ-hTERT cells should still retain a functional genotoxic damage response that does not involve senescence.

Most quantitative apoptosis assays measure an aspect of apoptosis at a particular point in time. However, these assays may not be useful to detect low-dose effects of genotoxic agents. For example, in this study we show that exposure to 3 μM Na$_2$CrO$_4$ does not allow for detection of a statistically significant change in mitochondrial depolarization (Fig. 2) or caspase 3 activity (Fig. 4) at a single time point 24 h after exposure. Nonetheless, this dose causes a decrease in clonogenicity to 2.4% of the control cells (Fig. 5) and an apoptotic loss of cells from the population over time (Fig. 6). Thus, at low doses, the changes in mitochondrial membrane potential and caspase activity may occur gradually, followed by a protracted increase in apoptosis. The overall response may account for a significant decrease in clonogenic survival, but a marked change in any single indicator of apoptosis at a given moment in time would not be expected. Our results show that it is important to conduct a dynamic population study and measure cumulative cellular outcomes within an exposed population when evaluating the effects of genotoxic agents.

As shown in Figs. 5, 6, and 7, the ultimate fate of a population of Cr(VI)-exposed cells, as defined by the proportion of cells within the population that undergo clonogenic survival, terminal growth arrest, or apoptosis (CGA index), depends upon dose. At low doses (1 μM Na$_2$CrO$_4$), a transient cell cycle arrest is followed by a relatively high rate of replicative growth recovery. Of the cells that have exited the cell cycle, the major cell fate is terminal growth arrest, and only a minor fraction of the cells, if any, undergo apoptosis. At a moderate dose (2–3 μM Na$_2$CrO$_4$), the apoptotic threshold has clearly been crossed, and the percentage of cells that regain replicative potential is low. The apoptotic fraction at 3 μM is virtually unchanged compared with cells exposed to 2 μM Na$_2$CrO$_4$. The change in the CGA index between these two doses shows a proportional shift from clonogenic survival to terminal growth arrest without a major shift to apoptosis. These results suggest that terminal growth arrest is the primary route of cellular elimination from the replicative population at threshold genotoxic exposures.

The population response to the lower doses is consistent with a p53-mediated transient checkpoint arrest followed by a trifurcate cellular fate (CGA), with terminal growth arrest as the dominate mode of exit from replicative competence (Fig. 8A). At higher doses, there is only a minimal fraction of clonogenic survivors, suggesting that the repair machinery activated during the initial checkpoint arrest encountered severe DNA damage. Nevertheless, although the clonogenic fraction at these doses is small, dividing strains do emerge
after a considerable recovery period. One possibility is that the progenitor "survivor" cells remained in an extended, but not terminal, form of growth arrest while they slowly repaired the damage. Alternatively, exposures such as these may induce and/or select for cells that are intrinsically resistant to apoptosis and/or terminal growth arrest. If the former is true, then the surviving population may be either normal (the product of successful repair) or genotypically altered (the product of incomplete or inaccurate repair). If the latter is true, then the surviving population may be more predisposed to neoplastic progression as a function of intrinsic resistance to replicative death. This population may also harbor unrepaird or inaccurately repaired DNA damage. Fig. 8B shows a model for the concept of selection of a fraction of cells with resistance to apoptosis and/or terminal growth arrest at relatively higher genotoxic exposures.

The fraction of cells that survived relatively high doses of Cr(VI) may have some intrinsic difference in the apoptotic regulatory pathway relative to the majority of cells. We have shown previously that Chinese hamster ovary (CHO) cells that survive a Cr(VI) apoptotic challenge may have a selective advantage in their ability to maintain mitochondrial stability (61). Cr(VI)-treated CHO cells cotreated with cyclosporin A, which is able to attenuate the mitochondrial permeability transition, experienced a reduced level of cyt c release, decreased apoptosis, and increased clonogenicity. Here, we show that Cr(VI)-induced apoptosis involves mitochondrial disruption in normal human fibroblasts, as indicated by an increase in mitochondrial depolarization (Fig. 2) and the release of cyt c from the mitochondria to the cytosol (Fig. 3). Thus, it is possible that the fraction of cells that survive when exposed to high doses of Cr(VI) may be able to better maintain mitochondrial stability and prevent cyt c release from the mitochondria. This would disable the apoptotic pathway in these cells despite the molecular apoptotic signals conveyed by the DNA damage. It is also possible that the survival fraction possesses some intrinsic difference in the regulation of terminal growth arrest. However, it is unlikely that these cells have resistance to either checkpoint arrest or cell cycle blockade because they are p53 competent (38), and no outgrowth of cells is observed until at least 8 days after exposure.

In summary, cell populations exposed to a genotoxin may have a different spectrum of responses depending on the extent of DNA damage. Our model predicts that relatively low genotoxic doses may cause low to moderate levels of DNA damage, which triggers a p53-mediated transient checkpoint arrest, presumably to allow an opportunity for DNA repair before replication. Cells in which DNA repair is incomplete will be eliminated from the replicative population. On the other hand, cells exposed to higher genotoxic doses that lead to excessive DNA damage may undergo a cell cycle blockade meant to prevent cells from reentering the cell cycle. In general, cells are eliminated from the replicative population by apoptosis or terminal growth arrest. The minor fraction of cells that survive the genotoxic insult may have a selective survival advantage over the majority of the population because of an intrinsic resistance to apoptosis and/or growth arrest. Unfortunately, this surviving fraction is the precursor pool from which neoplastic variants will emerge. Furthermore, these cells may harbor deleterious genotypic alterations caused by the genotoxic insult that eliminated the rest of the cells from the replicative population. Thus, these cells may be predisposed to further progression toward a neoplastic phenotype.

Materials and Methods

Cell Culture. BJ fibroblasts and the TERT-transfected clone BJ-hTERT (Geron Corp.) were maintained in DMEM/Medium 199 (4:1) containing 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) and 5 μg/ml gentamicin (Life Technologies, Inc., Gaithersburg, MD). Additionally, BJ-hTERT medium contained 10 μg/ml hygromycin B (Life Technologies, Inc.) as a selection agent for the hTERT transgene. Cells were incubated in a 95% air/5% CO2 humidified atmosphere at 37°C, and the medium was replaced every 48 h.

Treatment of Cells with Chromium. Sodium chromate (Na2CrO4·4H2O; J. T. Baker Chemical Co., Phillipsburg, NJ) was dissolved in double-distilled H2O and sterilized through a 0.2 μm filter before use. Cells were treated with a final concentration of 0–6, 9, or 12 μM sodium chromate for 24 h in complete medium. After 24 h, the cells were rinsed with PBS, the medium was replaced, and the cells were incubated for an additional 24 h before biochemical analysis.

Phosphatidylserine Translocation Assay. This assay was conducted as described previously (12). Briefly, BJ-hTERT cells were seeded at 103 cells/60-mm2 dish and incubated for 24 h prior to sodium chromate exposure. After the sodium chromate treatment, cells were gently harvested by trypsinization and combined with nonadherent cells from the culture medium. The cells were centrifuged at 600 × g for 5 min. Cell pellets were washed once in PBS and resuspended in 100 μl of binding buffer [10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 1.8 mM CaCl2] containing 2 μl of Annexin(V)-FLUOS (Roche, Indianapolis, IN). Samples were incubated in the dark at room temperature for 15 min. Thirty μl were loaded on a microscope slide, and the percentage of Annexin(V)-FLUOS-stained cells was determined using an Olympus AX70 microscope (Olympus, Lake Success, NY) with a fluorescent filter set suitable for FLUOS analysis (excitation at 460–490 nm and emission at 515 nm).

Mitochondrial Membrane Depolarization Analysis. BJ-hTERT cells were seeded at 7 × 105 cells/100-mm dish and incubated for 24 h before sodium chromate exposure. After the sodium chromate treatment, cells were gently harvested with trypsin and washed in PBS, and 105 cells were incubated with the mitochondrial membrane potential (∆Ψmt)-sensitive dye, JC-9 (Molecular Probes) at a final concentration of 5 μM for 10 min at 37°C. Cells were washed in PBS, and changes in ∆Ψmt were analyzed by flow cytometry using a minimum of 104 cells/sample (FACScan; Becton Dickinson, San Jose, CA). Mitochondrial membrane depolarization was indicated by an increase in green fluorescence at 530 nm. Both H2O2 (0.5 mM for 15 min) and valinomycin (5 μM for 24 h) were used as positive controls.

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Immunohistochemical Subcellular Localization of cyt c. BJ-hTERT cells were seeded on eight-well chamber slides (Lab Tek International, Naperville, IL) at $8 \times 10^3$ cells/well and incubated for 24 h before sodium chromate exposure. After the sodium chromate treatment, cells were fixed with methanol at $-20^\circ$C for 10 min and washed twice with ice-cold PBS. The cells were then rehydrated with PBS and then incubated with $10\%$ BSA in PBS for 30 min at room temperature. The cells were then rinsed twice with PBS and then incubated with primary mouse anti-cyt c monoclonal antibody (PharMingen, San Diego, CA) diluted 1:200 in PBS with $3\%$ BSA at 4°C overnight. The cells were then rinsed in PBS with $3\%$ BSA six times and incubated with goat ALEXA 488-conjugated antimouse IgG secondary antibody (Molecular Probes) diluted 1:800 in PBS with $3\%$ BSA for 1 h. The cells were then rinsed in PBS with $3\%$ BSA six times and observed with a CMIA Bio-Rad MRC 1024 confocal laser scanning microscope (Bio-Rad, Hercules, CA) at 488 nm fluorescence excitation and 520 nm fluorescence emission.

Caspase 3 Activity Analysis. BJ-hTERT cells were seeded at $5 \times 10^4$ cells/75-cm$^2$ flask and incubated for 24 h prior to sodium chromate exposure. After the sodium chromate treatment, cells were harvested by cell scraping and combined with nonadherent cells from the culture medium. Caspase 3 activity was determined using the FluorAce apoptotic activity kit (Bio-Rad). Cells were centrifuged at $600 \times g$ for 5 min. Cell pellets were rinsed once in PBS and resuspended in 100 $\mu$l of ice-cold apoptotic lysis buffer [10 mM HEPES (pH 7.4), 2 mM EDTA, 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, supplemented with protease inhibitors (10 $\mu$g/ml pepstatin A, 10 $\mu$g/ml aprotinin, and 20 $\mu$g/ml leupeptin; Sigma Chemical Co., St. Louis, MO)]. The cell suspensions were vortexed gently and then frozen/thawed five times by transferring from an isopropanol-dry ice bath to a 37°C water bath. The suspensions were then centrifuged at $12,000 \times g$ for 30 min at 4°C to pellet cell debris. Protein concentrations of the resulting supernatants were determined with the DC Protein Assay II (Bio-Rad). Samples containing an equal amount of protein were diluted to 1 ml and transferred to wells of a CoStar 48-well dish (Corning, Corning, NY). Ten $\mu$l of apoptotic substrate (Ac-DEVD-AFC; Bio-Rad kit) were added to each sample and control. The samples were gently mixed, and the fluorescence was determined using a Cytofluor 4000 fluorescence multiwell plate reader (PE Biosystems, Foster City, CA; excitation, 320–400 nm; emission, 505–555 nm). Fluorescence readings were taken at $t = 0$, $t = 60$ min, and $t = 120$ min. The amount of apoptotic fluorescent reaction product (AFC) was determined by linear regression of an AFC standard curve (per manufacturer’s instructions). Caspase 3 units were determined as $\Delta$AFC/min/$\mu$g protein $\times 1000$, and changes in caspase 3 activity were expressed as the fold increase in units of caspase 3 compared with control.

Clonogenicity Analysis. BJ-hTERT cells were seeded at $10^5$ cells/60-mm dish and incubated for 24 h prior to sodium chromate exposure. After the 24-h exposure, cells were collected by trypsinization, counted, and reseeded at $7 \times 10^3$ cells/60-mm$^2$ dish in triplicate. The plates were incubated for 12–13 days and then rinsed with PBS and incubated with crystal violet stain (80% methanol, 2% formaldehyde, and 2.5 g/l crystal violet) for 15–30 min at room temperature. The plates were thoroughly rinsed with distilled H$_2$O and allowed to dry. Colonies were counted, the triplicates were averaged, and clonogenicity was determined as a percentage of control.

Cell Growth Curves. BJ-hTERT cells were seeded at $10^5$ cells/60-mm dish and incubated for 24 h prior to sodium chromate exposure. A group of eight replicate dishes were seeded for each dose tested, and all of the replicates within the group received the same treatment. One replicate was taken every day over the 8-day time course and counted to determine total cell number at each dose and time. Cells were then harvested with trypsin and centrifuged at $600 \times g$ for 5 min, and the cell pellets were resuspended in 1 ml of PBS. Total cell number was determined using a Coulter Multisizer II cell counter (Coulter, Luton, United Kingdom).

Cellular Fate Analysis. The percentages of clonogenic survivors, growth-arrested cells, and apoptotic cells in populations exposed to 0–6 $\mu$m sodium chromate were compared at each dose tested. The clonogenic survivor fraction was determined by clonogenicity analysis as described. The apoptotic fraction (cell loss) was determined by subtracting the total number of cells remaining at the lowest point on the cell growth curve from the total number of cells at day one after seeding. [Previous EM studies in normal human fibroblasts showed that cell loss after Cr(VI) exposure was nearly 100% by apoptosis (30).] The remaining fraction of cells was assumed to be growth arrested and was confirmed by microscopic analysis. The percentages of each cell fate was expressed as the CGA index.

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References


