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Effect of Carcinogenic Chromium(VI) on Cell Death and Cell Cycle in Chinese Hamster Ovary Cells

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ABSTRACT **: Chromium compounds are known human and animal carcinogens. In this study, the effects of sodium chromate on apoptosis and cell cycle were investigated in order to unveil the elements of early cellular responses to the metal. Using Chinese hamster ovary cells (CHO-K1-BH4), we found that chromium (VI) treatment induced apoptosis in these cells, as signified by nuclear fragmentation, DNA laddering on agarose gel electrophoresis, and an increased proportion of cells with hypodiploid DNA. Preceding these changes, chromium(VI) treatment increased caspase 3 protease activity and also increased expression of p53 protein, while the level of bcl_2 protein was not changed. Coincubation with caspase inhibitor, Z-DEVD-FMK, inhibited chromium-induced apoptosis. In the flow cytometric analysis using propidium iodide fluorescence, an increase of cell population in G2/M phase was shown in cells exposed to at least 160 μM of sodium chromate for 72 h, from 9.8% for 0 μM chromium(VI) to 26.4% for 320 μM chromium(VI). Taken together, these findings suggest that chromium(VI)-induced apoptosis is accompanied by G2/M cell cycle arrest, and that p53-mediated pathway may be involved in positive regulation of G2/M arrest and a concurrent apoptosis in CHO cells.**

Key words : **Chromium(VI), Apoptosis, Cell cycle arrest, p53 protein, bcl_2 protein, Hypodiploid**

Introduction

Chromium is ubiquitous in the environment, occurring naturally in soils, rock and living organisms. Chromium exists in primarily two valence states, trivalent [chromium(III)] and hexavalent [chromium(VI)]. Chromium(III) and chromium (VI) are produced by many different industries including welding, chrome plating, chrome pigmenting, the ferrochrome industry and leather tanning (Anderson, 1981; Fishbein, 1981). Chromium(III) is unstable to enter cells but chromium(VI) enters into cells through membrane anionic transporters. Intracellular chromium(VI) is metabolically reduced to the ultimate chromium(III). It is estimated that several hundred thousand workers are potentially exposed to high levels of chromium(VI). The biological effects associated with chromium(VI) exposure are diverse and depend upon metal species. Exposure to only certain chromium(VI)-containing compounds is associated with an increased risk of lung cancer. Although chromium(III) is the ultimate DNA binding species of chromium within cells, it is non-carcinogenic because of its instability to pass through transporters residing within the cell membrane (Salinkow *et al.*, 1992). Chromium(VI) compounds are human carcinogen. They are potent inducers of tumors in experimental animals, and can neoplastically transform cells in culture (Fan and Harding-Barlow, 1987; De Flora and Wetterhahn, 1989). Chromium(VI) has been shown to induce a variety of types of DNA damage, including DNA single-strand breaks, alkali-labile sites, and DNA-protein cross-links in cultured mammalian cells (Tsapakos *et al.*, 1983; Hamilton and Wetterhahn, 1986). Chromium binding to DNA and non-histone nuclear proteins has been observed in rat liver and kidney tissues *in vivo* (Cupo and Wetterhahn, 1985) and in cultured cells (Wedrychowski *et al.*, 1985; Miller and Costa, 1988, 1989) after chromium(VI) treatment, as well as in mouse liver chromatin treated with chromium(III) *in vitro* (Ohba *et al.*, 1985).

DNA damage is an ever-present stress to most organisms during their life. The integrity of the genetic materials of living cells is continuously threatened by

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structural alterations caused by numerous genotoxic agents. Cells respond to DNA damage by activating DNA damage-inducible genes whose products contribute to apoptosis, cell cycle arrest, and DNA repair (Fritsche *et al.*, 1993; Smith and Fornace, 1996). Imbalance between cell growth and cell death has been proposed to be involved in tumor formation (Thompson, 1995). Accordingly, in response to various types of DNA damage, the cell cycle checkpoints and cell death signal are activated to stop cell growth and to eliminate multiplication of the genetically altered cells. Some cells arrest in G1 or G2 phase of the cell cycle. Two checkpoints in cell cycle, G1 and G2, play a very important role in the regulation of cells proceeding to S and M phases, respectively (O'Connor, 1997). Damaged cells stop DNA replication at G1 or G2 phase, presumably allowing the repair system to function before the next round of cell cycle. Alternatively, activation of the apoptotic cell death pathway is a safeguard to remove unrepairably damaged cells. It seems that cell proliferations and apoptosis are intrinsically linked, if not in all situations, at least in many. Several studies have suggested an intricate relationship between apoptosis and cell cycle (King and Cidlowski, 1995; Kasten and Giordano, 1998). However, it is yet unclear whether cell cycle arrest is a prerequisite for the activation of the apoptotic process.

Chromium(VI) has been shown to induce apoptosis in cultured cells (Blankenship, 1994). The dual potentials of chromium in carcinogenesis and apoptosis indicate that uncontrolled cell proliferation and programmed cell death may coexist in response to chromium-induced DNA damage. However, the apoptotic potential of chromium has inconsistently reported among various culture systems, suggesting that cellular response to chromium is varied and dependent on selected conditions and cells (Wang *et al.*, 1999; Carlisle *et al.*, 2000). Research on the mechanism of chromium(VI)-induced apoptosis has revealed multiple possibilities, but the exact pathways have not been determined. Current research has focused on two major pathways involved in chromium(VI)-induced apoptosis; p53-dependent and -independent pathways (Wang *et al.*, 1999; Ye *et al.*, 1999; Carlisle *et al.*, 2000). However, the underlying mechanisms are currently being examined.

In the present study, we examined cellular responses in chromium(VI)-treated Chinese hamster ovary cells (CHO-K1-BH4), including cell death, the distribution of cells in various phases of cell cycle, and p53 expression.

Materials and Methods

Chemicals

Sodium chromate (Na_2CrO4) was purchased from Aldrich Chemical Inc (Milwaukee, WI, USA).

Cell culture

Chinese hamster ovary cells, CHO-K1-BH4, were kindly provided by Dr Paul Donovan of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD, USA). The cells were maintained in Ham's F-12 medium (Biofluids, Rockville, MD, USA) supplemented with 5% fetal bovine serum albumin, 1 mM glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml (GIBCO BRL, Rockville, MD, USA). Cultures were maintained at 37°C in a humidified air containing 5% CO_2. The cells were seeded in 10 cm diameter culture plates, 1×10^6 cells/plate. 24 h prior to the treatment with sodium chromate, freshly dissolved in the above medium. Plates were used for treatments with 0, 40, 80, 160, 320 μM of sodium chromate. The cells were incubated with sodium chromate for 72 h. They were then harvested with 1 ml trypsin (0.25% w/v, Biofluids) for DNA flow cytometry and DNA fragmentation analysis.

Assessment of apoptosis

For the analysis for DNA fragmentation, the floating and attached cells from each plate were pooled in a 1.5 ml tube and incubated with 100 μl of lysis solution (5% Triton X-100, 10 mM ethylenediaminotetraacetic acid and 10 mM Tris-HCl, pH 8.0) on ice for 10 min. The lysate was then centrifuged at 16,000 g for 20 min at 4°C, and the supernatant was incubated with DNase-free RNase (0.4 mg/ml) at 37°C for 1 h, followed by 1 h at 37°C with proteinase K (0.4 mg/ml). The DNA was precipitated with 1/10 volume of 5 M NaCl and 1 volume of isopropanol. The DNA ladder was resolved in a 1.6% agarose gel and visualized by ethidium bromide (0.5 μg/ml) staining. For determination of nuclear fragmentation, pelleted cells were stained with acridine orange (4 μg/ml) and ethidium bromide (4 μg/ml) and then examined with a fluorescence microscope (Carl Zeiss). A minimum of 200 cells was counted microscopically and the proportion of apoptotic cells was expressed as a percentage of total cells.

Flow cytometry

The trypsinized cells were pelleted by centrifugation at 500 g for 5 min at 4°C and fixed in 70% ethanol for 24 h at −20°C. After fixation, cells were suspended in 0.5 ml PBS and counted with a hemocytometer. The cells (approximately 1×10^6 cells/ml) were then incubated with DNase-free RNase (0.1 mg/ml, Sigma) and propidium iodide (50 μg/ml, Sigma) at 4°C for at least 1 h prior to flow cytometric analysis. The propidium iodide-stained cells were assayed at 488 nm on an EPICS profile flow cytometer (Coulter, Hialeah, FL, USA) equipped with an air-cooled 20 mW argon laser. Minimums of 10,000 cells were collected in each run. The cells were selected by pulse-height (doublet elimination) analysis and only the integrated signals were collected to reject doublets. All histograms were evaluated by Multicycle software (Advanced version, Phoenix Flow Systems, San Diego, CA).

Measurement of caspase 3 enzyme activity

Activation of caspase 3 was quantified with the CaspACE Assay System according to the manufacturer's protocol (Promega, Madison, WI, USA). Cells were lysed with four cycles of freezing and thawing in 25 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, and 1X complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN, USA). After centrifugation for 20 min at 12,000 g, the amount of protein in the supernatant was determined BCA protein assay kit (Pierce, Rockford, IL, USA). For each sample, 30 μg of cell cytosolic extract was preincubated with DMSO vehicle or the CPP32/caspase-3 inhibitor (Ac-DEVD-CHO) for 30 min at 37°C and then incubated with the caspase 3-selective enzyme substrate (Ac-DEVD-AMC) for 1 h at 37°C. The fluorescence of the released 7-amino-4-methyl coumarin was measured using an excitation wavelength of 340 nm and an emission wavelength of 450 nm (CytoFluor II; Perspective Biosystem, Framingham, MA, USA). Caspase 3 activity was calculated by subtracting the amount of nonspecific enzyme activity detected in the presence of the Ac-DEVD-CHO inhibitor.

Western blot analyses of p53 and bcl_2

Cells were lyzed with RIPA buffer (1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml

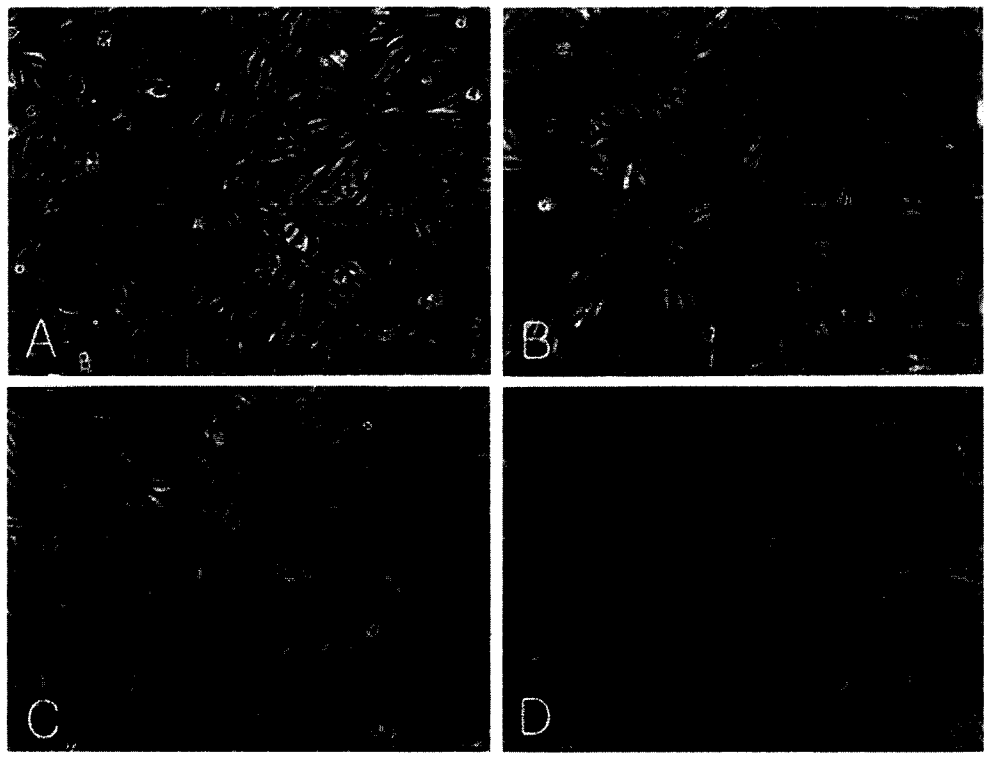

Fig. 1. Morphological changes in Chinese hamster ovary cells treated with 0 (A), 80 (B), 160 (C), and 320 μM (D) sodium chromate for 72 h.

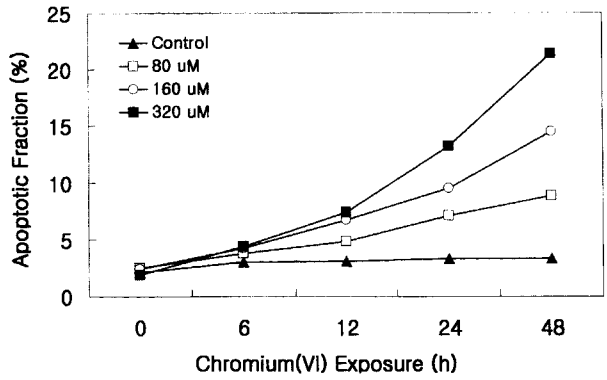

Fig. 2. Induction of apoptosis in Chinese hamster ovary cells exposed to sodium chromate as assessed by differential staining with acridine orange and ethidium bromide. Values are the means of two independent experiments.

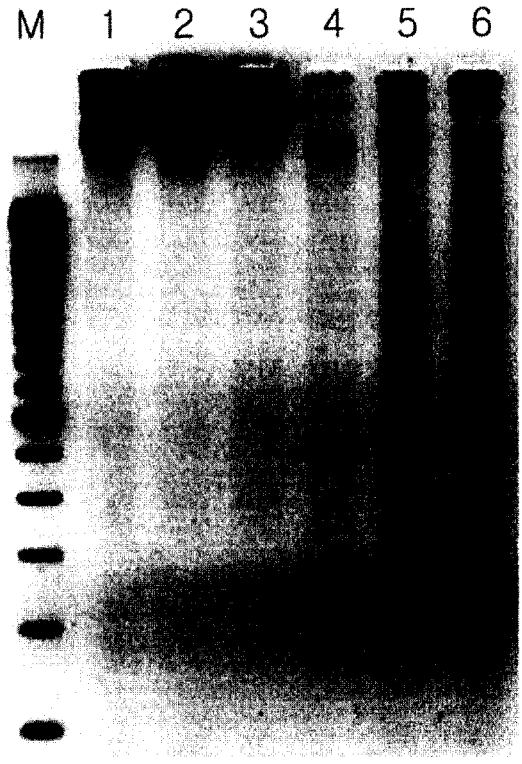

Fig. 3. Evidence of apoptosis appearing as DNA ladder in agarose gel electrophoresis of Chinese hamster ovary cells treated with 0, 40, 80, 120, 160, and 320 μM sodium chromate for 48 h.

phenylmethylsulfonyl fluoride) on ice for 1 h. Cell lysate containing 50 μg of protein was separated on 8% SDS-polyacrylamide gel (Novex, San Diego, CA, USA) and electrotransferred on to a nitrcellulose membrane (Trans-Blot, 0.45 μm pore size; Bio-Rad, Hercules, CA, USA). The membrane was incubated for 1 h at room temperature with 1 : 2000 diluted mouse anti-p53 monoclonal antibody (Pab 240, final concentration 500 ng/ml; Pharmingen, San Diego CA, USA) or anti-bcl_2 polyclonal antibody (Santa cruz Inc, CA, USA) in PBS containing 0.1% BSA. Horseradish peroxide-conjugated goat anti-mouse IgG was applied at a dilution of 1 : 4,000 in PBS/0.4% BSA for 1 h. The signal was visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham Life Science, Cleveland, OH, USA)

Results

The majority of chromium(VI)-treated CHO cells became elongated and resembled fibroblasts when the concentration of chromium(VI) was ≥ 160 μM (Fig. 1). The elongation developed in the 48 h of chromium(VI) exposure and persisted throughout the remaining 24 h of treatment. At the end of treatment, several characteristic features of the cells were recorded. During the 72 h exposure to 0-80 μM chromium(VI), the cell reached ~95% confluence. In contrast, at the same time cells treated with 320 μM chromium(VI) proliferated more slowly, and reached only ~60% confluence. The number of floating cells was proportional to the concentration of sodium chromate.

To determine whether chromium(VI) induced apoptosis in this cell line, we examined the effect of chromium(VI) treatment on hallmarks of apoptosis. Quantitation of nuclear fragmentation by differential staining with acridine orange and ethidium bromide showed that chromium(VI)-induced apoptosis was concentration-dependent. The effect of chromium(VI) treatment on apoptosis also was chromium(VI) concentration-dependent (Fig. 2). The DNA ladder, indicative of apoptosis, was observed in gels from cells treated with 160 μM sodium chromate for 48 h, and its intensity increased with rising concentration of sodium chromate (Fig. 3). Similarly, treatment with chromium(VI) increased the proportion of cells with hypodiploid amounts of DNA in a concentration-dependent fashion, as determined by flow cytometry (Fig. 4). The occurrence of a sub-G0/G1 peak, indicative of apoptosis, was detected in cultures treated with 160 and 320 μM chromium(VI).

In the flow cytometry analysis, chromium(VI) treatment for 72 h revealed the percentage of cells in G2/M phase tend to increase with rising concentration of chromium(VI). In a sample that contained the pooled attached/floating cells, the proportion of CHO cells in

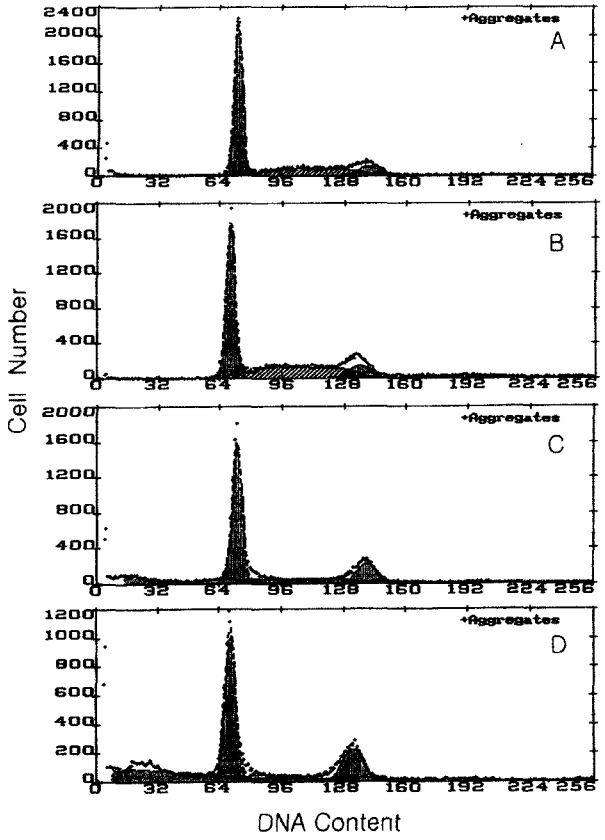

Fig. 4. Cell cycle analysis of Chinese hamster ovary cells after treatment with 0, 80, 160, and 320 μM sodium chromate for 72 h.

G2/M phase was significantly increased in cells exposed to at least 160 μM of chromium(VI) from 59.1% of cells in the G0/G1 phase, 31.1% in the S phase, and 9.8% in the G2/M phase for 0 μM chromium(VI), to 62.9%, 10.7%, and 26.4% for 320 μM chromium(VI) (Fig. 4). 160 μM chromium(VI) was sufficient to induce G2/M arrest when the cells were treated for 72 h. The percentage of cells at G2/M phase was dose-dependent, and thus more cells were stopped at the G2/M phase when cells received 320 μM chromium(VI).

Caspases are the known downstream effector of apoptosis. To determine whether caspase activity is increased by chromium treatment of CHO cells, caspase 3 activity was assessed in cell cytosolic extracts. Treatment with various concentrations of chromium for 48 h induced the increase in caspase 3 activity. Activation of caspase 3 activity by 320 μM chromium(VI) showed a peak activity at 24 h (Fig 5). In addition, preincubation of CHO cells with Z-DEVD-FMK, irreversible caspase inhibitors, blocked the chromium(VI)-

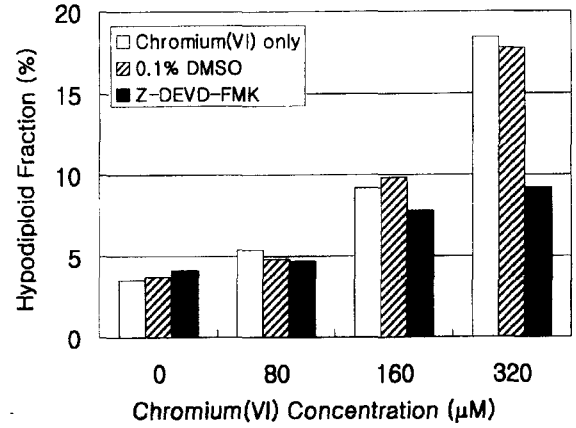

Fig 5. Blockade by caspase inhibitor of sodium chromate-induced apoptosis in Chinese hamster ovary cells. After a 30-min preincubation with medium alone, 0.1% DMSO vehicle, and 100 μM Z-DEVD-FMK, cells were coincubated with indicated concentrations of sodium chromate for 24 h. The proportion of cells with hypodiploid DNA was determined by flow cytometry. The data are the means of two independent experiments.

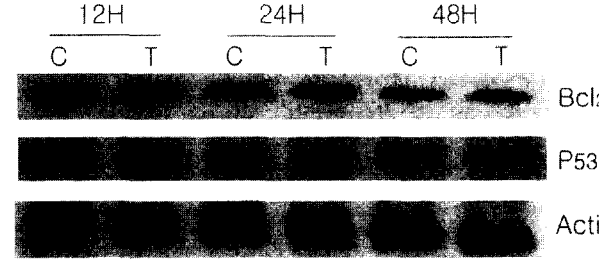

Fig. 6. Western blot analysis of p53 and bcl_2 protein expression in Chinese hamster ovary cells treated with 320 μM of sodium chromate for 12 h, 24 h, and 48 h. C, untreated cells; T, chromium(VI)-treated cells.

induced apoptosis. Preincubation with Z-DEVD-FMK before incubation with various concentrations of chromium(VI) for 24 h markedly inhibited the chromium(VI)-induced increase in sub-G0/G1 peaks, as determined by flow cytometry (Fig 5).

To find the possible effector molecules responsible for chromium-induced apoptosis and G2/M cell cycle arrest, expression of p53 and bcl_2, which are known marker proteins on apoptotic process or cell cycle regulation, were assayed by Western blot analysis after chromium treatment. The results showed that there was no significant difference in the level of bcl_2 protein among cells cultured with 320 μM chromium(VI) for 12, 24, and 48 h. However, following exposure for 24 h, significant up-regulation of p53 protein was noticed (Fig. 6).

Discussion

The results obtained from this study revealed that cell proliferation and viability were inhibited as the concentration of chromium(VI) in the medium increased. This cell cycle block and cell death occurred at high concentration of chromium(VI) as high as 80 μM. Several metallic compounds have been reported to cause cell cycle and growth inhibition. For example, beryllium blocks normal human lung fibroblast at G0/G1/pro-S stage (Lehnert *et al.*, 2001). Lithium causes delayed mitotic arrest of mouse neuroblastoma cells (Garcia-Perez *et al.*, 1990). Nickel and zinc arrest chinese hamster ovary and human prostate carcinoma cells at G2/M phase, respectively (Shiao *et al.*, 1999; Liang *et al.*, 1999). In these cases, delayed cell growth frequently accompanies apoptosis.

A G2/M block often occurred when cells are subjected to the challenge of various DNA damaging agents. The most well known example is the G2/M block caused by radiation (Hwang and Muschel, 1998). The delay of cell proliferation is considered necessary for the repair of damaged DNA (Zhou and Elledge, 2000). Mammalian cell systems prevent carcinogenesis against genotoxic stress by at least two different mechanisms, i.e., by evoking cell cycle arrest, eliciting DNA repair systems, and eliminating cell through induction of apoptosis. The concurrence of G2/M arrest with apoptosis in response to DNA damaging agents indicates that a common signal is sent to prevent damaged cells from further replication and to eliminate them. Concurrence of these cellular responses has been associated with p34cdc2 kinase inhibition by dephosphorylation (Demarcq *et al.*, 1994). Also, the progression of cells through mitosis after the exposure to DNA damaging agents has been suggested to be stalled by p53 protein. However, the molecular mechanism has not been clearly determined. Such a signal is not detectably associated with the level of p53 protein expression. This is consistent with a report that expression of p53 in cells from nasal biopsies is not different between nickel workers and control population. A similar activation of G2/M arrest and apoptosis have been also observed in cisplatin-treated CHO cells (Demarcq *et al.*, 1994).

Upon further examination of a molecular mechanism in chromium(VI)-induced apoptosis, we observed a up-regulation of p53 protein at $\geq$ 160 μM of chromium(VI) but had no effect on the bcl_2 protein, suggesting that this mode of apoptosis follows a p53-dependent pathway. This also provides an evidence for the observation that chromium(VI) blocks entry of replicating cells into mitosis in previous studies (Xu *et al.*, 1996). So, we may hypothesize that minor population of chromium-damaged cells escaped from apoptotic eradication may further mutate and eventually undergo neoplastic transformation.

It was shown that chromium(VI) induces the activation of p53 protein in CHO cells, human lung epithelial, and human lung fibroblast cells (Blankenship *et al.*, 1994; Wang *et al.*, 1999; Carlisle *et al.*, 2000). The reduction of chromium(VI) mediates the generation of free radicals (Shi *et al.*, 1999). These free radicals can then cause DNA damage, which activates upstream kinases that phosphorylate p53 protein (Ashcroft *et al.*, 1999). Although p53 plays a role, at least in part, in chromium-induced apoptosis, the induction of free radicals can also lead to apoptosis by other pathways. Chromium(VI)-induced apoptosis due to the mitochondrial damage has been reported but the findings are not consistent. Chromium(VI)-induced apoptosis, which is blocked by cyclosporin A, was accompanied by mitochondrial instability. Unlike cyclosporin A, Z-VAD-FMK, a broad-spectrum protease inhibitor that blocks caspases, did not decrease apoptotic cells, suggesting a caspase-independent pathway (Pritchard *et al.*, 2000). In contrast, it was shown that caspase 3, but not caspase 1, plays a role in chromium(VI)-induced apoptosis (Singh *et al.*, 1998). Regardless of the discrepancy concerning caspases, this study has shown that chromium(VI) treatment increased the activity of caspase 3 in CHO cells. Chromium(VI)-induced apoptosis was also blocked by nonselective caspase inhibitor such as Z-DEVD-FMK (Fig 5).

Although further studies directly measuring ROS generation under the same culture conditions are required, it is possible that the effect of chromium(VI) on apoptosis may be due to direct damage of the mitochondria by generation ROS (Ryberg *et al.*, 1990). This rationale is supported by the report that chromium(VI) that escapes reduction in the cytoplasm is taken up by the mitochondria damaging and causes injuries to both DNA and proteins (Singh *et al.*, 1998). Further investigations seem to be necessary to determine the cause of this contradiction.

In summary, we observed a concentration-dependent effect of chromium(VI) on the morphology, cell cycle and apoptosis in CHO cells. These cellular responses

are most likely induced by common effectors. P53-mediated signaling pathway may be involved in positive regulation of both G2/M arrest and apoptosis in CHO cells.

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