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Nickel (II)-induced apoptosis and G2/M enrichment

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Abbreviations: PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; CHO, Chinese hamster ovary

Abstract

Treatment with certain DNA-damaging agents induce a complex cellular response comprising pertubation of cell cycle progression and/or apoptosis on proliferating mammalian cells. Our studies were focused on the cellular effects of nickel (II) acetate, DNA-damaging agent, on Chinese hamster ovary (CHO) cells. Fragmented DNAs were examined by agarose gel electrophoresis and cell cycle was determined by DNA flow cytometry using propidium iodide fluorescence. Apparent DNA laddering was observed in cells treated with 240 μM nickel (II) and increased with a concentration-dependent manner. Treatment of nickel (II) acetate resulted in apoptosis which was accompanied by G_2/M cell accumulation. Proportion of CHO cells in G_2/M phase was also significantly increased in cells exposed to at least 480 μM nickel (II) from 57.7% of cells in the G_0/G_1 phase, 34.7% in the S phase, and 7.6 % in the G_2/M phase for 0 μM nickel (II), to 58.6%, 14.5%, and 26.9% for 640 μM nickel (II). These findings suggest that nickel (II) can modulate cellular response through some common effectors involving in both apoptotic and cell cycle regulatory pathways.

Keywords: DNA damage, apoptosis, cell cycle, flow cytometry, Sub-G1, DNA ladder

Introduction

The critical balance between cell proliferation and death is imperative for the survival of multicellular organisms. Apoptosis, or programmed cell death, is an active, genetically controlled process which allows an organism to eliminate unwanted cells through a safe, orderly process (Wyllie, 1980). Apoptosis is characterized morphologically by cell shrinkage, apoptotic body formation, chromatin condensation and fragmentation, and biochemically by cleavage of DNA into oligonucleosomal DNA fragments. The latter process results in DNA ladders on agarose gel electrophoresis (Walker *et al*., 1995).

In many cultured cell lines, apoptosis can be readily triggered by DNA-damaging agents (Allday *et al*., 1995), γ-irradiation (Sellins and Cohen, 1991), activation of Fas/Apo1 receptor (Watanabe-Fukanaga *et al*., 1992), or some anticancer agents (Lowe *et al*., 1993). Activation of the cell death pathway is a safeguard to remove unrepairably damaged cells. Cells also can be restricted to a particular phase of cell cycle for the entry into apoptosis, although this cell cycle dependence does not appear to be a general process. Damaged cells stop DNA replication at G_1 (Dulic *et al*., 1994) or G_2 phase (O'Connor *et al*., 1993). The arrest at the G_1 and G_2 checkpoints indicates the necessity of the cells to gain time to repair the damaged DNA before the next round of cell cycle. It seems that cell proliferation and apoptosis are intrinsically linked, if not in all situations, at least in many. Recent studies have suggested an intricate relationship between apoptosis and cell cycle (King and Cidlowski, 1995). However, it is yet unclear whether cell cycle arrest is a prerequisite for the activation of the apoptotic process.

In an effort to find the link between cell proliferation and cell death, we first examined the induction of apoptosis and the cell cycle distribution in Chinese hamster ovary (CHO) cells treated with different quantities of nickel (II) acetate which induces genotoxic effects such as DNA-protein crosslinking, DNA strand breaks, sister chromatid exchange, and oxidative DNA base damage (Kasprzak, 1995).

Materials and Methods

Cell culture and treatment

Chinese hamster ovary cell variant, CHO-K1-BH4, grew in a monolayer culture in Ham's F-12 medium (Biofluids, Inc., Rockville, MD) supplemented with 5% fetal bovine serum, 1 mM glutamine, 100 unit of penicillin/ml, and 100 μg of streptomycin/ml. Cultures were maintained at 37°C in a humidified air containing 5% CO_2. To eliminate the possible interfering factor such as confluency-induced apoptosis (Brezden and Rauth *et al*., 1996), the cells were differently seeded in 10-cm diameter culture

plates, 5×10^5 or 1×10^6 cells/plate, 24 h prior to the addition of nickel (II) acetate (Sigma, St. Louis, MO), freshly dissolved in the above medium. Plates seeded with 5×10^5 cells were used for treatments with 0, 40, 80, and 160 μM nickel (II) and plates seeded with 1×10^6 cells were used for treatments with 240, 360, 480, and 640 μM nickel (II). The cells were incubated with nickel (II) acetate for 24, 48, and 72 h. They were then harvested with 2 ml trypsin (0.25% w/v, without calcium and magnesium, Biofluids, Inc.) for DNA flow cytometry and DNA fragmentation assay.

Laser Scanning Microscopy

Cells were grown on coverslips for 3 days, and treated with 240 μM nickel (II) acetate for 3 days. The media were removed, and then the cells were stained by direct applying with acridine orange (Sigma, St. Louis, MO; 40 μg/ml in PBS) and ethidium bromide (Sigma; 20 μg/ml in PBS). Following washing once with PBS with special care to avoid the excessive cell loss, the coverslip was mounted on a slide and then observed with a LSM 410 laser scanning microscope (Carl Zeiss, Jena, Germany).

Analysis of DNA ladder for apoptosis

The analysis for DNA fragmentation was carried out by the method described by Gong *et al*. (1993). The floating and attached cells from each plate were pooled by centrifugation and fixed in 70% ethanol at -20˚C for 72 h. The cells were then centrifuged at 800g for 5 min and the cell pellets ($1\text{-}2\times10^6$) were resuspended in 40 μl of phosphate-citrate (PC) buffer, consisting of 192 parts of 0.2 M Na_2HPO_4 and 8 parts of 0.1 M citric acid (pH 7.8) for at least 30 min. After centrifugation at 1000g for 5 min, cell pellets were used in cell cycle analysis and the supernatant was transferred to new tube and concentrated by vacuum in Speed Vac concentrator for 20 min. A 5 μl of a solution of DNase-free RNase (Sigma; 0.4 mg/ml) was then added. After 1 h incubation at 37˚C, 5 μl of a solution of proteinase K (Sigma; 0.4 mg/ml) was added and extract was incubated for an additional 1 h at 37˚C. After the incubation, 12 μl of loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol) was added and the entire content of the tube was transferred to the gel. The DNA ladder was resolved in a 1.8% agarose gel and visualized by ethidium bromide (0.5 μg/ml) staining.

Flow cytometry

Cell pellets removed from the PC buffer described above were resuspended in 200 μl of Hanks' buffered salt solution (HBSS) 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) equipped with an air-cooled 20 mW argon laser. A minimum 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) (Kallioniemi *et al*., 1994). The G0/G1 and G2/M peaks were determined using Gaussian curves. Resolution of G_0/G_1 peaks was evaluated by coefficient of variation which is calculated by dividing the half-peak width by the mean channel number. The S phase fraction was calculated by the area under the zero order polynomial curve between mean channel number of G_0/G_1 peak and that of G2/M peak.

Statistical analysis

Statistical evaluation of the data was performed either by analysis of variance (ANOVA) or by Student's *t*-test for paired comparisons. Trends were examined by linear regression statistics using the SAS program. Differences with probability values ≤ 0.05 were considered to be statistically significant.

Results and Discussion

CHO cell morphology

Morphological changes in response to the treatment of CHO cells with nickel (II) acetate were examined by confocal microscopy after staining with acridine orange. Nuclear fragmentation, indicative of apoptosis, was apparent in 240 μM treated adherent cells at day 3 after treatment (Figure 1). Also at this time, 18% of the adherent cells exhibited pyknotic, fragmented nuclei. Phase-contrast microscopy of CHO cells treated with 480 μM nickel (II) acetate for 72 h showed an epithelial cell-like morphology of untreated CHO cells to be elongated and resembled fibroblasts (Figure 2). The elongation developed in the first 24 h of nickel (II) acetate and persisted throughout the remaining 72 h of exposure. The change in morphology of CHO cells has been reported to be associated with increased intracellular cAMP concentrations and activation of cAMP-dependent protein kinase (Costa, 1978).

DNA fragmentation assay

In initial assays of DNA fragmentation, the untreated control cells and cells treated with $\leq$ 240 μM nickel (II) acetate proliferated rapidly and reached 90-95% confluency 72 h after treatment. The doubling time of untreated cells was determined to be 14 ±1 h. In contrast, cells treated with 640 μM nickel (II) acetate proliferate more slowly, and reached only ~50% confluency. Fragmented

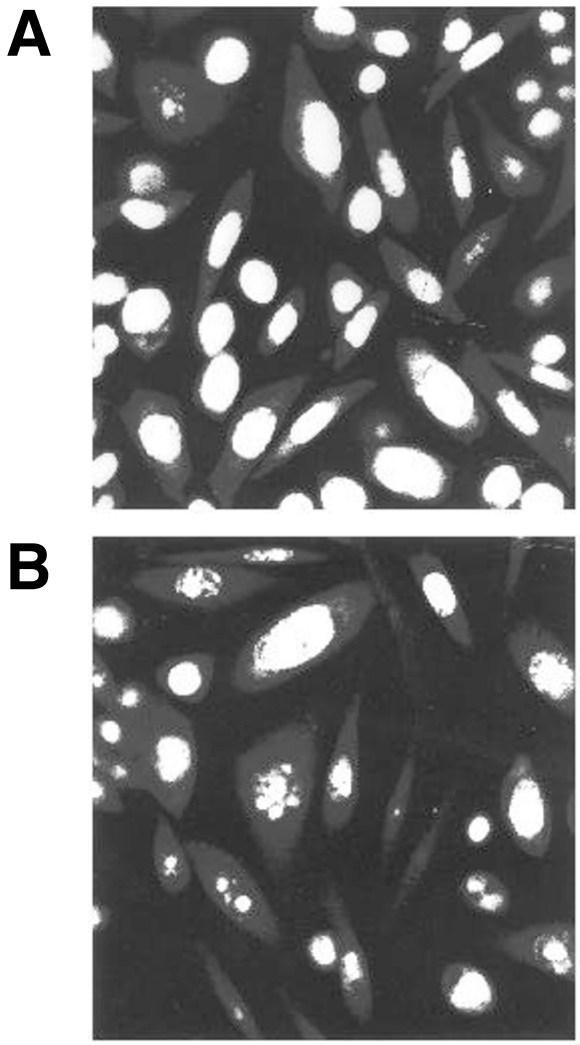

Figure 1. Confocal microscopic finding showing nuclear fragmentation as morphological criteria of apoptosis in nickel (II) acetate-treated CHO cells. Cells were grown on coverslips and treated with 240 (M nickel (II) acetate for 72 h. Morphology of untreated cells (A) and treated cells (B) was identified by acridine orange/ethidium bromide staining.

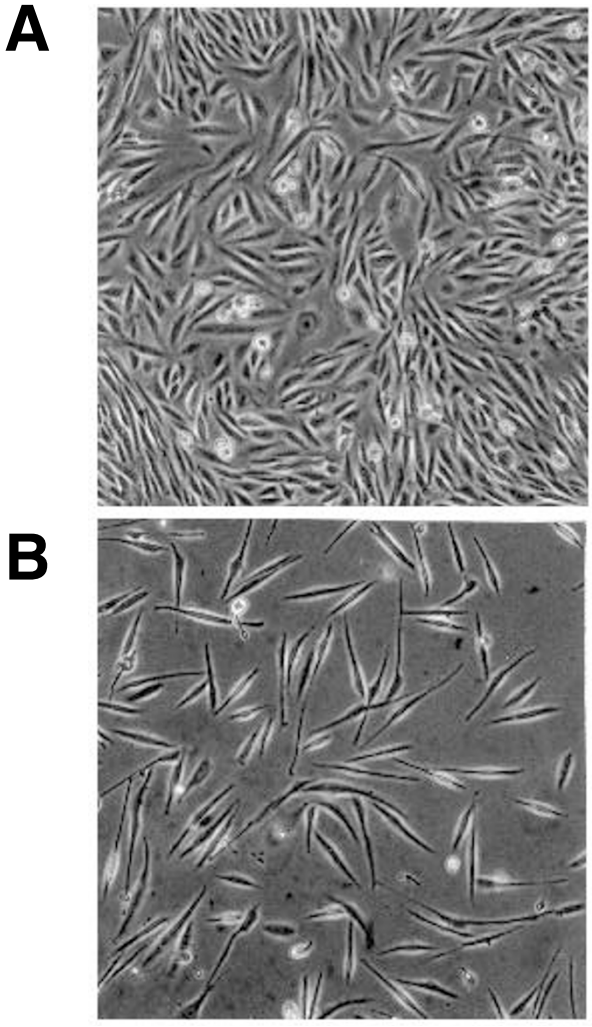

Figure 2. Phase-contrast microscopic finding showing morphological change in nickel (II) acetate-treated CHO cells: (A) untreated, (B) treated with 480 (M nickel (II) acetate for 72 h.

DNAs pooled from adherent and floating cells were analyzed by 1.8% agarose gel electrophoresis (Figure 3A). Internucleosomal DNA fragmentation was faintly detected in cells treated with 160 μM nickel (II) acetate, and its intensity increased markedly with the rising concentration of nickel (II) acetate.

Figure 3B shows an agarose gel electrophoresis of fragmented DNAs isolated from pooled nonadherent and adherent cells at various times after nickel (II) treatment. DNA ladder was faintly detected in 48 h of exposure. But it was clearly evident following 72 h nickel (II) acetate exposure. As determined in comparison with molecular weight marker DNA, the average size difference between the DNA fragments was approximately 180 bp (Orren *et al*., 1977). This fragmentation pattern is consistent with the molecular weight expected to result from the inter-nucleosomal DNA cleavage associated with apoptotic cell death. Nickel (II)-induced apoptosis has been once reported but the findings are not consistent with our results. Myeloid precursor cell line HL-60, but not murine fibro-blasts L929 nor gingival fibroblasts, showed an indication of apoptosis after exposure to 1 mM nickel (II) (Schedle *et al*., 1995). These differences probably could be cell-specific and/or associated with nickel (II) concentration.

Cell cycle analysis

Cell cycle analysis after nickel (II) acetate treatment for 72 h revealed the percentage of cells in the S phase

tended to decrease and that in G_2/M phase tended to increase with the increasing nickel (II) concentration (Figure 4). As revealed by trend analysis, those overall tendencies were significant with $P < 0.001$. The decrease in the S phase cell proportion was statistically significant at ≥ 320 μM nickel (II), and the increase in the G_2/M phase cells, indicative of an arrest, was significant in samples treated with ≥480 μM nickel (II) acetate ($P < 0.01$). The occurrence of a sub-G_1 peak, typical of apoptosis (Darzynkiewicz *et al*., 1992), was detected in cells treated with 480 ≥ μM nickel (II) acetate and its peak increased with the rising nickel (II) concentration. Table 1 shows that untreated CHO cells for 72 h displayed a typical distribution of 57.7% in G_0/G_1 phase, 34.7% in the S phase, and 7.6% in G_2/M phase. But the treatment of cells with 640 (M nickel (II) acetate for 72 h increased the number of cells in G_2/M to 26.9% whereas the number of cells in the S-phase decreased 14.5%. Cell cycle distributions in 24 and 48 h were also examined following nickel (II) treatment (Data not shown). But, proportion of CHO cells in each phase didn't showed significant change, from 55.7% in the G_0/G_1 phase, 25.0% in the S phase, and 19.3 % in the G_2/M phase of cells treated with 0 μM nickel (II) for 48 h, to 66.6%, 15.9%, and 17.5% for 640 μM nickel (II).

In our experiments, we observed the obvious dose-dependent accumulation of cells in the G_2/M phase. Also, the data for apoptosis showed a dose-dependent occurrence. Thus, it seemed that the cells underwent nickel (II)-induced apoptosis during or after mitotic failure and consequently, prevented the cell cycle progression through G_1 and S phase. However, we could not rule out the possibility that the apoptosis is occurring in the cells arrested in G_2/M phase. The concurrence of G_2/M cell accumulation with apoptosis in response to nickel (II) indicates that a common signal is sent to prevent damaged cells from further replication and to eliminate them. DNA damage results in arrest of cells in the G_1

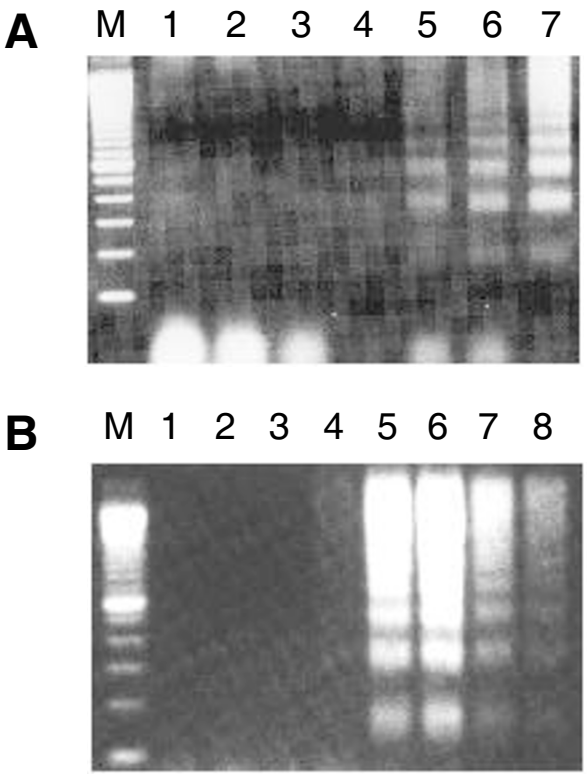

Figure 3. Internucleosomal DNA fragmentation induced by nickel (II) acetate. (A) DNA extracted with phosphate citrate buffer from untreated and treated CHO cells was analyzed by 1.8% agarose gel electrophoresis. Lanes: 1, untreated for 72 h; 2, 40 (M; 3, 80 (M; 4, 160 (M; 5, 240 (M; 6, 480 (M; 7, 640 (M nickel (II) acetate treatment. (B) Time course of apoptosis appearing as DNA ladder in cells treated with 480 (M nickel (II) acetate. Lanes 1, untreated; 2, 12 h; 3, 24 h; 4, 48 h; 5, 72 h; 6, 4 day; 7, 5 day; 8, 6 day after treatment; M, 100 bp DNA molecular weight marker.

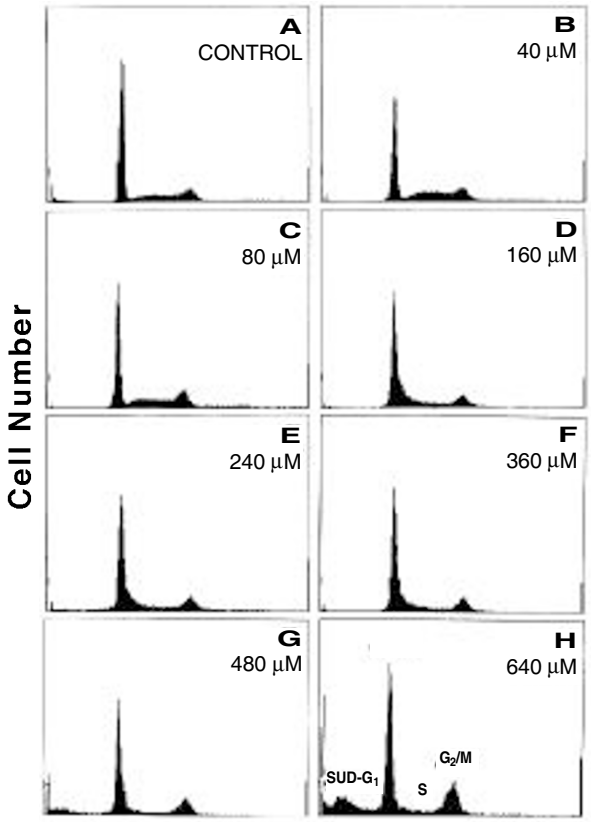

Figure 4. Cell distribution at G1, S, and G2/M phases among nickel (II) acetate-treated cells. Propidium iodide-stained cells were analyzed by EPICS profile flow cytometry to determine the cell cycle distribution. (A) untreated, (B) treated with 40 (M, (C) 80 (M, (D) 160 (M, (E) 240 (M, (F) 360 (M, (G) 480 (M, and (H) 640 (M nickel (II) acetate for 72 h.

Table 1. Cell cycle distribution of CHO cells treated with different concentrations of nickel (II) acetate.

Nickel (II) (μM)	G_0/G_1%	S %	G2/M %
0	57.7 ± 3.5	34.7 ± 3.1	7.6 ± 1.5
40	53.8 ± 4.8	36.8 ± 2.7	8.7 ± 1.5
80	52.4 ± 3.1	35.3 ± 5.2	12.2 ± 2.5
160	51.1 ± 4.8	38.7 ± 5.1	10.1 ± 0.5
240	61.7 ± 4.7	28.7 ± 2.1	9.5 ± 3.6
360	64.2 ± 3.0	23.2 ± 2.1	12.5 ± 3.9
480	59.4 ± 2.8	16.3 ± 3.0	24.2 ± 5.3
640	58.6 ± 2.8	14.5 ± 3.2	26.9 ± 0.6

(Dulic *et al*., 1994) and G_2 phases (Sit and Chen, 1997) of the cell cycle through mechanisms involving the G_1 and G_2 phase checkpoints, respectively. Damaged cells stop DNA replication at G_1 or G_2 phase, presumably allowing cells to repair DNA lesions prior to mitosis. Apoptosis is also triggered in response to various DNA damage. A similar activation of G_2/M arrest and apoptosis have been also observed in a number of report (Kim *et al*., 1993; Demarcq *et al*., 1994; Smith and Fornace, 1996). Concurrence of these cellular responses has been associated with $p34^{cdc2}$ kinase inhibition by dephosphorylation (Lock and Ross, 1990). However, the molecular mechanism has not been clearly determined. The progression of cells through mitosis after the exposure to DNA damaging agents has been suggested to be facilitated by p53 protein, whereas p53-deficient cells are stalled in G_2 thereby triggering apoptosis (Guillouf *et al*., 1995; Stewart *et al*., 1995; Wahl *et al*., 1996). Also, other proteins involved in cell cycle can be associated with both apoptosis and G_2/M arrest. Further investigations seem to be necessary to find out the common effector(s).

In summary, we have demonstrated that nickel (II) acetate, DNA damaging agent, induced apoptosis and G_2/M cell accumulation. Concurrence of these cellular responses suggests that common effector(s) may be activated to regulate cell growth and cell death in response to DNA damage.

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