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Apoptosis, Bcl2 Expression, and Cell Cycle Analyses in Nickel(II)-Treated Normal Rat Kidney Cells

Nickel compounds are carcinogenic to human and are potent inducers of kidney and lung tumors in experimental animals. In this study, the effects of nickel(II) acetate on apoptosis, cell cycle and bcl2 expression in normal rat kidney (NRK-52E) cells were investigated. Nickel(II) induced apoptosis in NRK-52E cells as demonstrated by DNA laddering. Apparent DNA laddering was observed in cells treated with 480 μM for 48 hr. In the flow cytometric analysis using propidium iodide fluorescence, an increase of cell proportion in G2/M phase was shown in cells exposed to at least 320 μM of nickel(II) acetate, from 7.7% for 0 μM of nickel(II) to 16.5% for 480 μM of nickel(II) acetate. Induction of apoptotic cell death by nickel(II) was accompanied by reduction of bcl2 protein expression, while the level of p53 protein was not changed. Taken together, our data indicate that nickel(II)-induced apoptosis in NRK-52E cells is accompanied by G2/M cell cycle arrest, regardless of p53 function, and that bcl2-mediated signaling pathway may be involved in positive regulation of nickel(II)-induced apoptotic cell death in NRK-52E cells.

Key Words : *Nickel(II); Apoptosis; Cell Cycle; Bcl2*

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INTRODUCTION

Nickel(II) is associated with lung cancers in human lung and kidney tumors in experimental animals (1). Nickel(II) has been found to produce diverse types of DNA damage, including DNA single-strand breaks, alkali-labile sites, and DNA-protein cross-link, in cultured cells and in the tissue of experimental animals (1, 2). 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 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 (3, 4).

There are two main ways of cell death, that is, necrosis and apoptosis. In contrast to necrosis, apoptosis results from the perturbation of a physiological cell environment and causes plasma membrane blebbing, cell volume loss, nuclear condensation, and endonucleolytic degradation of DNA at nucleosomal intervals. The latter process results in DNA ladders on agarose gel electrophoresis. Apoptosis is an important process by which unwanted cells are deleted from the tissues of multicellular organism in order to prevent genomic instability and cellular dysfunction (5-7). Alternatively, some cells arrest in G1 and/or G2 phase of the cell cycle. Two checkpoints in the cell cycle, G1 and G2, play a very important role in the regulation of cells proceeding to S and M phases, respectively. Damaged cells stop DNA replication at G1 or G2 phase, presumably allowing the repair systems to function before the next round of cell cycle (8-11). What determines whether a particular cell should arrest or activate the suicide program is not fully understood. Concurrence of cell cycle arrest and apoptosis has been also observed in a number of reports (12-14). However it is not yet clear whether cell cycle arrest is a prerequisite for the activation of the apoptotic process.

Here, we examined cellular responses in nickel(II) acetate-treated NRK-52E cells, including cell death, the distribution of cells in various phases of cell cycle, and the expression of p53 and bcl2 proteins.

MATERIALS AND METHODS

NRK-52E cell lines used in this study was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). NRK-52E cells were maintained as adherent cells Dulbecco Modified Eagle's Medium (DMEM)

supplemented with 10% fetal bovine serum, 1 mM of glutamine, 100 units of penicillin/mL and 100 μg of streptomycin/mL. Logarithmically growing cells were treated with 0, 160, 320, and 480 μM of nickel(II) acetate for 72 hr for cell cycle analysis, and with 480 μM of nickel(II) for 0, 6, 12, 24, 48, and 72 hr for the analysis of DNA ladder and protein expression.

Trypsinized cells were pelleted by centrifugation at 500 *g* for 10 min and fixed in 70% ethanol for 24-72 hr at -20℃. Cells (~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℃ for at least 1 hr prior to flow cytometric analysis. The propidium iodide-stained cells were assayed at 488 nm on an EPICS Profile cytometer (Coulter, Hialeah, FL, U.S.A.) equipped with an air-cooled 20 mW argon laser. All histograms were evaluated by Multicycle software (Advanced version; Phoenix Flow Systems, San Diego, CA, U.S.A.).

The method used for the analysis of DNA fragmentation representative of apoptosis, which appears as a ladder in agarose gel electrophoresis, was modified from Subramanian et al. (15).

For protein analysis of p53 and bcl2, cells were lyzed with RIPA buffer (1× phosphate buffered saline [PBS], 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/mL phenylmethylsulfonyl fluoride) on ice for 1 hr. Cell lysates containing 60 μg of p53 and bcl2 proteins were separated on 8% and 12% Tris-glycine SDS-polyacrylamide gel (Novex, San Diego, CA, U.S.A.), respectively, and then electrotransferred onto a nitrocellulose membrane (Trans-Blot, 0.45 μm pore size: Bio-Rad, Hercules, CA, U.S.A.). The membrane was incubated for 1 hr at room temperature with 1:2,000 diluted mouse anti-p53 monoclonal antibody (Pab 240, final concentration 500 ng/mL; PharMingen, San Diego, CA, U.S.A.) or anti-bcl2 polyclonal antibody (Santa Cruz Inc, CA, U.S.A.) in PBS containing 0.1% bovine serum albumin. Horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Inc.) was applied at a dilution of 1:6,000 in PBS/0.4 % BSA for 1 hr. The signal was visualized using an Enhanced Chemiluminescence (ECL) detection kit (Amersham Life Science, Cleveland, OH, U.S.A.).

RESULTS

To study nickel(II)-induced cellular response, we used NRK-52E cells, epithelial-like cells derived from normal rat kidney, the main target of nickel(II) toxicity and carcinogenicity. In the flow cytometric analysis, nickel(II) acetate treatment for 72 hr revealed the percentage of cells in G2/M phase tend to increase with rising concentration of nickel(II) acetate (Fig. 1). In a sample that

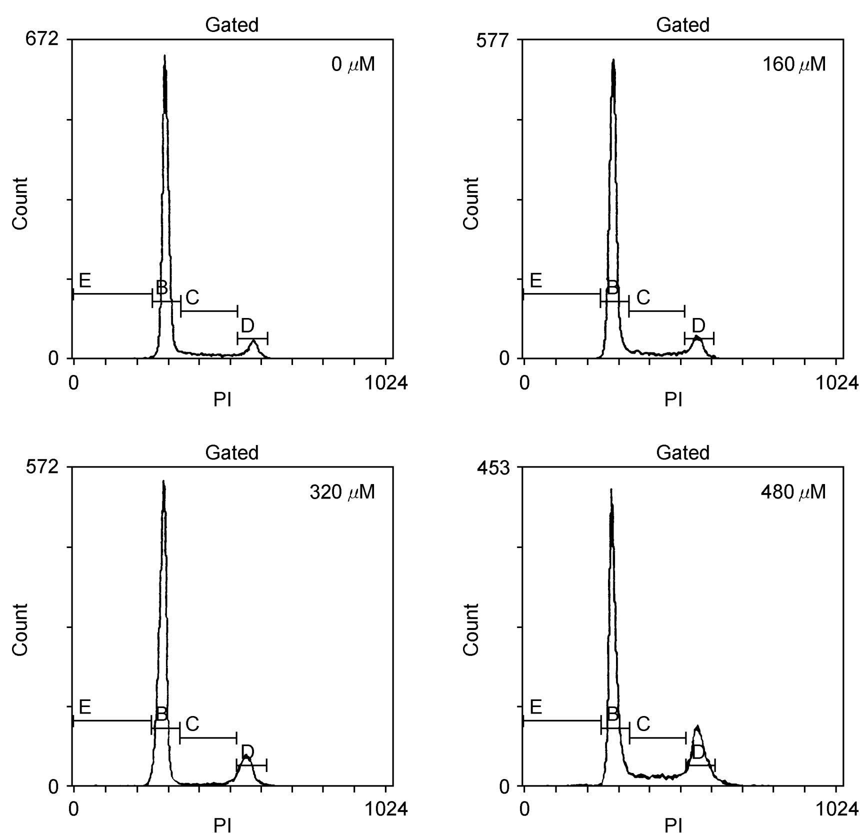

Fig. 1. Cell cycle analysis of normal rat kidney cells after treatment with different concentrations of nickel(II) acetate for 72 hr. Fractions B, C, and D indicate cells in G0/G1, S, and G2/M phases, respectively (PI, propidium iodide).

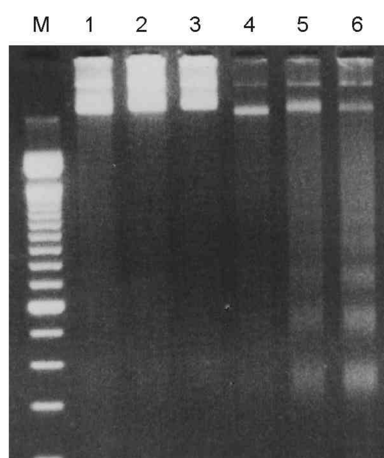

Fig. 2. Evidence of apoptosis appearing as a DNA ladder in agarose gel electrophoresis for normal rat kidney cells treated with 480 μM of nickel(II) acetate. Lanes: 1, untreated for 72 hr; 2, 6 hr; 3, 12 hr; 4, 24 hr; 5, 48 hr; 6, 72 hr after treatment; M, 100 bp DNA molecular weight marker.

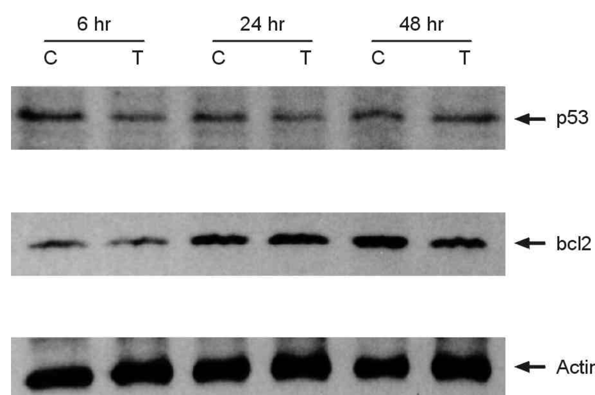

Fig. 3. Western blot analysis of p53 and bcl2 protein expression in normal rat kidney cells treated with 480 μM of nickel(II) acetate for 6 hr, 24 hr, and 48 hr. C, untreated cells; T, nickel(II)-treated cells.

contained the pooled attached/floating cells, the proportion of NRK-52E cells in G2/M phase was significantly increased in cells exposed to at least 320 μM of nickel(II) from 77.2% of cells in the G0/G1 phase, 15.1% in the S phase, and 7.7% in the G2/M phase for 0 μM of nickel(II), to 76.8%, 6.7%, and 16.5% for 480 μM of nickel(II) acetate (data not shown). The percentages of cells in each phase were calculated from two experiments. The DNA ladder was observed in gels from cells treated with 480 μM of nickel(II) acetate for 48 hr, and its intensity increased markedly following 72 hr exposure. The DNA ladder was rarely detectable in the first 24 hr (Fig. 2). To find possible effector molecules responsible for the apoptosis and G2/M arrest, expressions of p53 and bcl2, which are known marker proteins on apoptotic process or cell cycle regulation, were assayed by Western blot analysis after nickel(II) treatment. The results showed that there was no significant difference in the level of p53 protein among cells cultured with 480 μM of nickel(II) for 0, 6, 24, and 48 hr. However, following exposure for 48 hr, significant down-regulation of bcl2 protein was noticed (Fig. 3).

DISCUSSION

Exposure to nickel(II), either by inhalation or ingestion (16), may induce multiple genotoxic effects, such as DNA single-strand breaks, cross-linking of DNA-protein, sister chromatid exchange and oxidative DNA base damage, including the promutagenic 8-oxo-2'-deoxyguanosine (17). 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 by eliminating damaged cells through induction of apoptosis.

Nickel(II)-induced apoptosis was first reported in Chinese hamster ovary (CHO) cells, which were not known to be the target cells for nickel(II) toxicity or carcinogenicity (4). The presence of DNA ladder in agarose gel, indicative of apoptosis, in NRK cells treated with $\geq$320 μM but not with lower concentrations of nickel(II) acetate, might support the notion of threshold in nickel(II) dose to induce the apoptosis. Among the important proteins that regulate apoptosis induced by a variety of stimuli are bcl2 family and p53 proteins. Some proteins within bcl2 family, e.g., bcl2 and bclx inhibit apoptosis, while others such as Bax and Bak promote it (18). In fact, bcl2 can prevent or delay apoptosis induced by a variety of stimuli, including growth factor deprivation, free radicals, alteration in Ca^{++} concentration, cytotoxic lymphokines, some types of virus, radiation, and most chemotherapeutic drugs, suggesting that bcl2 controls the common final pathway involved in cell death regulation (19, 20). It has been shown that bcl2 may protect cancer cells from apoptosis induced by a variety of anticancer agents and contribute to neoplastic cell expansion by prolonging cell survival (21). In NRK-52E cells, we found that nickel(II) treatment decreased the expression of bcl2 protein but had no effect on the expression of p53 protein. The decrease of bcl2 protein expression in response to 480 μM of nickel(II) acetate confirms the role of bcl2 in apoptotic cell death. Further studies on bcl2-mediated apoptotic signaling pathway in response to nickel(II) treatment are in progress in our laboratory.

In addition to the decrease in DNA replication, NRK-52E cells damaged by nickel(II) tend to choose G2/M checkpoint, allowing the repair systems to function be-

fore the next round. Cell proliferation and apoptosis seem to be intrinsically linked, in some situations, if not all. A variety of molecules including pRb, p53, cdks, and c-myc are working on these processes (12). p53 protein responds to DNA damage either by enforcing cell cycle arrest or by triggering apoptosis (22). Given these reports, however, the observation in our study that no significant change in p53 protein expression by Western blot analysis suggests that G2/M cell cycle arrest occurred through the p53-independent pathway.

In summary, we have demonstrated that nickel(II) acetate induced apoptosis and G2/M cell accumulation in NRK-52E cells and that it might directly activate the bcl2-mediated apoptotic signaling pathway, through which it triggers a fail-safe mechanism designed to eliminate cells that have been subjected to the potentially mutagenic agents.

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