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Soluble and insoluble nickel compounds exert a differential inhibitory effect on cell growth through IKK α -dependent cyclin D1 down-regulation

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Abstract

It is well-known that insoluble nickel compounds possess much more potent carcinogenic activities as compared with soluble nickel compounds. Although it is assumed that the different entry and clearance rate are responsible for the difference, the mechanisms underlying the different carcinogenic activities are still not well understood yet. In the present study, we found that exposure to soluble, but not insoluble nickel compounds, caused a significant inhibition of cell growth and G1/G0 cell cycle arrest, which was concomitant with a marked down-regulation of cyclin D1, an essential nuclear protein for controlling G1/S transition, while both soluble and insoluble nickel compounds showed similar effects on NF κ B activation, HIF-1 α protein accumulation and TNF- α transcription and CAP43 protein expression at same doses range. The down-regulation of cyclin D1 is due to protein degradation rather than inhibition of transcription, because the nickel compounds treatment did not change cyclin D1 mRNA level, while MG132, the proteasome inhibitor, can rescue the degradation of cyclin D1 caused by soluble nickel compound. Moreover, the soluble nickel-induced cyclin D1 degradation is dependent on its Thr286 residue and requires IKK α , but not HIF-1 α , which are both reported to be involved in cyclin D1 down-regulation. Taken together, we demonstrate that soluble, but not insoluble nickel compound, is able to cause cyclin D1 degradation and a cell growth arrest in an IKK α -dependent manner. Given the role of cyclin D1 and cell proliferation in carcinogenesis, we anticipate that the different effects of soluble and insoluble nickel compounds on cyclin D1 degradation and cell growth arrest may at least partially account for their different carcinogenic activities.

Keywords

nickel compounds; cell growth arrest; cyclin D1; HIF-1 α ; IKK α

Introduction

Nickel, a widely distributed natural metal, is industrially applied in alloys and industrial processes such as electroplating, foundries, catalysts, battery manufacturing, and coinage (Oller et al., 1997; Sunderman, 1989). Based on their water solubility, nickel compounds are categorized as soluble and insoluble forms. Epidemiological studies have shown that long-

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term inhalation exposures of workers employed in nickel refining operations to mixtures of soluble and insoluble nickel compounds in nickel refineries have been associated with excess lung cancer and nasal sinus cancer (1990; IARC, 1990). The carcinogenic activity of nickel compounds has been shown to inversely correlate to its solubility in water (National-Toxicology-Program, 1996a; National-Toxicology-Program, 1996b; National-Toxicology-Program, 1996c; Oller, 2002; Oller et al., 1997). The ability of insoluble nickel compounds to induce cancer formation has been verified by a series of animal carcinogenicity studies with different exposure routes in different research groups. Ottolenghi et al. has reported that the inhalation of insoluble nickel compounds induces adenomas and adenocarcinomas in the lungs of male and female F344 rats (Ottolenghi et al., 1975). Intramuscular (i.m.) injection of nickel compounds into male F344 rats induced sarcomas at the injection site (Sunderman and Maenza, 1976). Kasprzak et al. has showed that insoluble nickel compound is carcinogenic when injected into Wistar rats (Kasprzak et al., 1983). The U.S. National Toxicology Program (NTP) has conducted nickel animal carcinogenic studies by the inhalation route, which is most relevant to human carcinogenesis, and showed that insoluble nickel compounds induce lung tumors in rats and mice, but soluble nickel compounds do not (National-Toxicology-Program, 1996a; National-Toxicology-Program, 1996b; National-Toxicology-Program, 1996c). Several oral exposure studies in rats, mice and dogs with soluble nickel compounds also failed to induce tumors (Oller, 2002; Schroeder and Mitchener, 1975; Schroeder et al., 1974). These lines of evidence clearly indicate that insoluble nickel compounds are potent carcinogens, while soluble nickel compounds are less potent. Accordingly, the insoluble nickel compounds are classified as a human carcinogen, while soluble nickel compounds are considered as “not classifiable as a human carcinogen” (ACGIH, 1998) or “the carcinogenicity of soluble nickel compounds cannot be determined” (Haber et al., 2000a; Haber et al., 2000b).

After entry to cells, nickel compounds have been demonstrated to promote the generation of ROS (Landolph, 1999); interact directly or indirectly with nucleic acids and cause genotoxic damage (Sunderman, 1993); induce epigenetic alterations including DNA methylation and loss of histone acetylation (Sutherland and Costa, 2003); and activate signaling pathways such as PI-3K/Akt (Li et al., 2004), HIF-1 α (Salnikow et al., 2000), NF- κ B (Chen et al., 2001; Cruz et al., 2004; Huang et al., 2002), AP-1 (Cruz et al., 2004; Huang et al., 2002) and NFAT (Huang et al., 2001; Lu et al., 2005). All these events are believed to relate to their carcinogenic activities. Although the different entry and clearance rate as well as the persistence of water-soluble and water-insoluble nickel compounds in cells are presumably thought to be responsible for their different carcinogenic activities (Oller et al., 1997; Seilkop and Oller, 2003), so far, the mechanisms under the difference remains to be fully defined.

Carcinogenesis results from a synergism between genotoxic and nongenotoxic factors (Hecker, 1987; Zoumpourlis et al., 2003). The former induce irreversible genetic alterations (tumor initiation), whereas the latter promote tumor development by favoring the clonally outgrowth of the genetically altered cells. It is believed that the growth of genetic mutated cells is indispensable during the process of carcinogenesis (Charnley and Wilson, 1991; Cohen and Ellwein, 1988). Thus, it is of interest to investigate whether soluble and insoluble nickel compounds can differently affect cell growth. In the present study, we demonstrated that soluble, but not insoluble nickel, is able to cause significant cell growth arrest and cyclin D1 degradation in an IKK α -dependent manner. These results provide the first evidence that the different effect of soluble and insoluble nickel compounds on cyclin D1 down-regulation and cell growth arrest may be associated with their different carcinogenic activities.

Materials and Methods

Cell Culture and Reagents

Human lung carcinoma A549 cells were cultured in Ham's F-12K medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 ig/ml streptomycin. Wild-type, IKK $\alpha^{-/-}$ (Devin et al., 2001), HIF1 $\alpha^{+/+}$ and HIF1 $\alpha^{-/-}$ (Ryan et al., 1998; Salnikow et al., 2000; Salnikow et al., 2003b) mouse embryo fibroblasts (MEFs) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Calbiochem, San Diego, CA). Human bronchial epithelial cell line HCCBE-3 (Ramirez et al., 2004) was cultured in defined keratinocyte serum-free medium (Invitrogen). The cultures were dissociated with trypsin and transferred to new 75-cm² culture flasks (Fisher, Pittsburgh, PA) after centrifuge from one to three times a week. FBS was purchased from Life Technologies, Inc.; DMEM was from Calbiochem (San Diego, CA); Nickel chloride (NiCl₂) was purchased from Aldrich (Milwaukee, WI); nickel sulfide (NiS) was purchased from Alfa (Ward Hill, MA); Nickel subsulfide (Ni₃S₂) was obtained from INCO (Toronto, Canada). Antibodies against cyclin D1, p21, caspase-3 and CDK4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against Phosphor-Cyclin D1 T286, ATF2 and IKK α were from Cell signaling Technology (Danvers, MA); RB antibody was from BD Biosciences (San Jose, CA); HIF-1 α antibody was from Novus Biologicals (Littleton, CO); FLAG and β -Actin antibodies were purchased from Sigma (St. Louis, MO).

Cell growth inhibition analysis

1 $\times$ 10³ of viable cells suspended in 100 μ l complete medium supplemented with 10% FBS were added to each well of 96-well plates. The plates were incubated at 37°C in a humidified atmosphere of 5% CO₂. 12 h later, the cells were exposed to nickel compounds for the times as indicated in figure legends. The exposed cells were taken pictures under microscopy, and then extracted with 50 μ l lysis buffer. The cell growth was measured by using a CellTiter-Glo[®] Luminescent Cell Viability Assay kit (Promega, Madison WI) with a luminometer (Wallac 1420 Victor2 multipliable counter system) as described previously (Ouyang et al., 2006b). The results are expressed as inhibition of cell growth, which was calculated as following:

$$\text{Inhibition of cell growth (\%)} = \frac{\text{luciferase activity of untreated cells} - \text{luciferase activity of treated cells}}{\text{luciferase activity of untreated cells}} \times 100$$

Cyclin D1 expression assay

2 $\times$ 10⁵ of cells were cultured in each well of 6-well plates to 85–90% confluence. After exposure to nickel compounds together with or without MG132, a chemical inhibitor of proteasome-dependent protein degradation, for different times as indicated in the figure legends. The treated cells were washed once with ice-cold PBS and then extracted with SDS-sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with specific antibodies against cyclin D1 and β -Actin which was used as a protein loading control. The protein bands specifically bound to primary antibodies were detected as indicated above.

Transfection and Western blot

293T cells were cultured in a 6-well plate until they reached 85–90% confluence. Five (5) μ g of FLAG-cyclin D1 T286A/pCMV5 expression vector, which was described in the previous study (Kwak et al., 2005) were mixed with 10 μ l of Lipofectamine 2000 reagent, and used to transfect each well in the absence of serum. After 6 h, the medium was replaced

with 10% FBS DMEM. Approximately 48 h after the beginning of the transfection, the cells were exposed to 0.5mM of NiCl₂ for 24 hrs, and then extracted for Western blot assay to detect cyclin D1 level as describe above with antibodies against FLAG, cyclin D1 and β -actin. β -actin was used as a protein loading control.

RT-PCR

2×10^5 of cells were cultured in each well of 6-well plates to about 85% confluence. After exposure to nickel compounds for 12h, the cells were extracted with Trizol reagent (Invitrogen, CA), and the total RNA was isolated following the manufacturer's instructions. 1 μ g of total RNA was reverse-transcribed (Superscript II, Invitrogen), and the cDNA was subjected to PCR amplifications using primer pairs specific for murine cyclin D1 (forward, 5'-gtg cca tcc atg cgg aa-3'; backward, 5'-gga tgg tct gct tgt tct ca-3'), for murine β -actin (forward: 5'-cat ccg taa aga cct cta tgc c-3'; backward: 5'-acg cag ctc agt aac agt cc-3'), for human cyclin D1 (forward: 5'-gag caa aga tgg cgc ata at-3'; backward: 5'-aag ttt gcc gtg gtg ttt ct-3') and human β -actin (forward: 5'-gcg aga aga tga ccc aga tca t-3'; backward: 5'-gct cag gag gag caa tga tct t-3'). The β -actins were used as the internal control. The PCR cycling conditions were: 25 cycles of 94 °C for 45 sec, 60 °C for 45 sec and 72 °C for 45 sec.

Cell cycle analysis

2×10^5 of cells were cultured in each well of 6-well plates to 70–80% confluence with normal culture medium. The cell culture medium was replaced with 0.1% FBS DMEM with 2 mM L-glutamine and 25 ig gentamicin and cultured for 24 h and then exposed to nickel compounds. The cells were harvested and fixed with 3 ml of ice-cold 80% ethanol overnight. The fixed cells were washed twice with PBS, and then suspended in propidium iodide staining solution (propidium iodide 50 μ g/ml, RNase A 10 mg/ml and 0.1% Triton X-100) (Sigma Chemical, St. Louis, MO) for at least 1 h at 4 °C. The DNA content was determined by flow cytometry using the Epics XL FACS (Beckman Coulter) and EXPO 32 software as described in our previous studies (Ouyang et al., 2006b; Ouyang et al., 2005).

Nuclear extraction

The cells were cultured in 100-mm dishes and treated with 0.5 mM nickel chloride for 6 h and 12 h. The nuclear proteins were extracted according to the protocol of Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA). In brief, cells were collected by centrifuge for 5 minutes at $600 \times g$, and lysized in 200 μ l of Cytosol Extraction Buffer A including DTT and protease inhibitors. Then 11 μ l of Cytosol Extraction Buffer B was added, and mixed by vortex vigorously for 5 seconds and incubated on ice for 1 min. The cell lysis was subjected to centrifuge immediately for 5 min at $16,000 \times g$. The supernatant was the cytoplasmic fraction. The crude nuclei pellet was resuspended in 100 μ l ice-cold Nuclear Extraction Buffer and agitated at high speed for 40 minutes by vortex, and subjected to centrifuge for 10 minutes at $20,000 \times g$. The supernatant was the nuclear extraction proteins.

Statistical Analysis

The significance of the difference between the treated and untreated groups was determined with the Student's t test. The results are expressed as mean $\pm$ S.D.

Results

Exposure of A549 cells to soluble nickel compound caused cell growth arrest and cyclin D1 degradation

Soluble and insoluble nickel compounds have been demonstrated to possess different carcinogenic activities (National-Toxicology-Program, 1996a; National-Toxicology-Program, 1996b; National-Toxicology-Program, 1996c; Oller, 2002; Oller et al., 1997). However, previous findings by us and others have also demonstrated that the soluble and insoluble nickel compounds at certain dosages have similar effects on various cellular events, including generation of intracellular ROS and activation of cell signaling pathways, which include HIF-1 α , NF κ B and PI-3K/Akt (Costa et al., 2005; Huang et al., 2002; Li et al., 2004). To compare the differential effects of soluble and insoluble nickel compounds on cell cycle and proliferation, we first determined equivalent doses of both types of nickel compounds by evaluation of their activation of cell signaling pathways in different doses. As shown in Figs. 1a–1c, NiCl₂ at doses ranged from 0.25 mM to 1 mM are equivalent to NiS at doses ranged from 0.25 μ g/cm² to 2 μ g/cm² in the activation of transcription factor NF κ B and HIF-1 α , and induction of TNF- α and CAP43 gene expression. In view of the role of cell growth in carcinogenesis, the lung being the primary target organ of nickel compounds, we examined the growth rate of human lung carcinoma A549 cells exposed to soluble and insoluble nickel compounds. As shown in Figs. 1d and 1e, the growth of A549 cells were significantly inhibited by the exposure of NiCl₂, a soluble nickel compound, but not NiS, an insoluble nickel compound. The growth inhibition caused by soluble nickel but not insoluble nickel was also found in other type of cells, namely human bronchial epithelial cell line HCCBE-3 (Ramirez et al., 2004) and mouse skin epidermal cell line JB6 Cl41 (Li et al., 2004; Ouyang et al., 2006a) (Figs 1f–1i), indicating that the differential growth inhibition effects of soluble but not insoluble nickel compounds is a general phenomena, not cell type specific.

The growth inhibition was concomitant with an arrest of cell cycle progression at G1/G0 phase (Fig. 2a). The cells exposed to NiCl₂ exhibited a significant increase in the proportion of G1/G0 phase, while with a marked decrease in the proportion of S phase (Fig. 2a). Since both NiCl₂ and NiS showed similar biological effects on HIF-1 α protein accumulation, NF κ B activation, TNF- α and CAP43 expression at the same doses used in the studies of cell cycle and growth, this rules out the possibility that differential effects of NiCl₂ and NiS on the inhibition of cell growth and cell cycle progression are due to either inactivation of insoluble nickel compounds or discrepancy of cytotoxicity to exposed cells.

Eukaryotic cell cycle progression is stringently regulated by a series of proteins. Cyclin D1 is one of the key regulators of G1/S transition (Serrano et al., 1993). To test whether the G1/G0 arrest in NiCl₂-exposed A549 cells is due to change of cyclin D1 protein level, we analyzed the cyclin D1 protein levels in the A549 cells exposed to nickel compounds. As shown in Fig. 2b, the treatment of A549 cells with NiCl₂ did cause a marked decrease in the cyclin D1 protein level, while the treatment of NiS only had a marginal effect. Consistently, the hypophosphorylation status of retinoblastoma (RB), the cyclin D1 downstream target which mediates G1/S phase transition by binding transcription factors such as E2F (Feliars et al., 2002), was observed following soluble nickel treatment, whereas insoluble exposure did not shown any inhibitory effects (Fig. 2b). Moreover, the other cell cycle regulating proteins, cyclin E and p21, were also detected. Cyclin E did not show any observed changes after nickel exposure, while p21 protein levels were increased by both nickel compounds even though insoluble nickel had much less effect (Fig. 2b). The decrease in the cyclin D1 protein levels seems to be caused by the proteasome-dependent proteolysis rather than the inhibition of the transcription, because the treatment of NiCl₂ did not affect cyclin D1 mRNA level (Fig. 2c), while the co-incubation of cells with MG132, a chemical proteasome

inhibitor, could reverse the down-regulation of cyclin D1 protein level caused by NiCl_2 (Fig. 2d). These results indicate that soluble nickel and insoluble nickel compounds have differential effects on the down-regulation of cyclin D1 expression mediated by a proteasome-dependent cyclin D1 degradation.

Exposure of MEFs to soluble nickel compound also caused cell growth arrest and cyclin D1 degradation

The Cyclin D1 protein degradation has been shown to be regulated by selective kinases and transcription factors. Since MEFs with specific targeted gene knockouts are optimal cell models for the investigation of the kinase and transcription factor involved in the down-regulation of cyclin D1 by NiCl_2 , we examined the cell growth, cell cycle progression and cyclin D1 expression level in the MEFs exposed to NiCl_2 . The results indicated that the treatment of MEFs with NiCl_2 , but not NiS , also caused a cell growth arrest (Figs. 3a and 3b), a G1/G0 arrest (Fig. 4a) and a marked decrease in cyclin D1 protein level (Fig. 4b), but did not affect its mRNA level (Fig. 4c). These results are consistent with the observations in A549 cells, further confirming the effect of soluble nickel compounds on cell growth and cyclin D1 degradation and suggesting that the effect may have no cell-type specificity again.

HIF-1 α is not involved in cyclin D1 degradation caused by soluble nickel compounds

Since NiCl_2 has a similar effect on cell growth and cyclin D1 down-regulation in MEFs and A549 cells, we employed the MEFs with specific gene deficiency to study the molecular mechanisms of the cyclin D1 degradation by NiCl_2 . Since HIF-1 α is a key transcription factor mediating the biological functions of nickel compounds (Li et al., 2004; Salnikow et al., 2000), which is also reported to be involved in the regulation of cyclin D1 stability, we first examined the potential involvement of HIF-1 α in the degradation of cyclin D1 upon NiCl_2 exposure. Consistent with previous reports, exposure of MEFs to both NiCl_2 and NiS caused a significant accumulation of HIF-1 α to a similar extent (Fig. 5a). Nonetheless, HIF-1 α was not required for the down-regulation of cyclin D1 in NiCl_2 -exposed cells because treatment of NiCl_2 caused a significant decrease in cyclin D1 in both HIF-1 $\alpha^{+/+}$ and HIF-1 $\alpha^{-/-}$ MEFs to a similar extent (Figs. 5b and 5c).

IKK α is required for cyclin D1 degradation and growth arrest caused by soluble nickel components

Phosphorylation at Thr286 and proteasomal degradation of cyclin D1 is considered as a prerequisite for normal progression of the S-phase (Diehl et al., 1997; Mukherji et al., 2008). Therefore, we detected cyclin D1 phosphorylation status following NiCl_2 treatment. As shown in Fig. 6a, NiCl_2 treatment in combination with MG132 could increase cyclin D1 phosphorylation at Thr286, while NiCl_2 alone down-regulated phosphorylation at Thr286, which might be due to the decreased total cyclin D1 expression. Most recently, Kwak et al. has reported that IKK α plays a role in cyclin D1 turnover through the phosphorylation at Thr286 (Kwak et al., 2005). In view of our result that nickel compounds can activate NF- κ B that is the downstream molecule of IKK complex (Chen et al., 2001; Cruz et al., 2004; Huang et al., 2002; Zandi et al., 1997), we examined the cyclin D1 protein levels in wild type (WT) and IKK $\alpha^{-/-}$ MEFs (Fig. 6b) after exposure to various concentrations of nickel compounds. The results showed that treatment of NiCl_2 caused a significant cyclin D1 decrease in WT MEFs, but did not cause any observed down-regulation of cyclin D1 in IKK $\alpha^{-/-}$ MEFs (Fig. 6c). Again, the treatment of NiS has only a marginal effect on the cyclin D1 levels in both WT and IKK $\alpha^{-/-}$ MEFs (Fig. 6c). Then we compared the subcellular distribution of cyclin D1 protein in WT and IKK $\alpha^{-/-}$ MEFs following nickel treatment, since it was reported that IKK α -dependent cyclin D1 phosphorylation is responsible for cyclin D1 protein translocation from nuclear to cytosol where it is subjected to degradation (Cao et al., 2001; Kwak et al., 2005). As shown in Fig. 6d, cyclin D1 located

mainly in cytosol rather than the nuclear part in both type of cells in absence of nickel treatment. Moreover, in WT cells nickel exposure triggered cyclin D1 protein decline in both cytosol and nuclear parts, which was blunt in $IKK\alpha^{-/-}$ cells. These results strongly indicate that $IKK\alpha$ is required for the degradation of cyclin D1 by $NiCl_2$.

Given that the regulation of cyclin D1 turnover by $IKK\alpha$ is through the phosphorylation of Thr 286 residue, we examined whether point mutation at T286A of cyclin D1 can prevent the degradation of cyclin D1 by $NiCl_2$. As shown in Fig. 6e, the treatment of $NiCl_2$ failed to cause the degradation of cyclin D1 with the point mutation though it caused marked endogenous cyclin D1 degradation. Consistently, the cyclin D1 protein degradation in both cytosol and nuclear parts was compromised by expression of cyclin D1 T286A mutant (Figs. 6d and 6f). Moreover, expression of cyclin D1 T286A mutant partially attenuated the growth inhibitory effect of $NiCl_2$ (Fig. 6g). It may be noted that overexpression of cyclin D1 T286A mutant did not reverse the growth inhibitory effects of $NiCl_2$ completely. The explanation for this might be due to either compromising effect of the endogenous cyclin D1 or other possibility that the growth inhibition by $NiCl_2$ is largely, but not entirely, mediated by cyclin D1 downregulation.

Consistent with the failure to induce cyclin D1 degradation, $NiCl_2$ did not induce an evident cell cycle arrest at G1/G0 phase in $IKK\alpha^{-/-}$ MEFs (Figs. 6h and 6i). It may be notable that untreated $IKK\alpha^{-/-}$ MEFs showed a much higher proportion of cells in the G1/G0 phase as compared with untreated WT MEFs (Figs. 6h and 6i), which seems contradictory to the higher stability of cyclin D1 protein level in $IKK\alpha^{-/-}$ MEFs. This may be explained by the findings that cyclin D1 level oscillates during the cell cycle progression (Stacey, 2003). Cyclin D1 is induced specifically during the G2 phase, remains high through mitosis and into the G1 phase, and then declines as the cells enter the S phase. Although cyclin D1 is known to be critical for the transition of cell cycle from the G0/G1 to S phase, cyclin D1 levels must be low during the S phase, and the decline is a fundamental requirement for normal cell cycle progression (Stacey, 2003). Another explanation for the high cell proportion of $IKK\alpha^{-/-}$ MEFs in the G0/G1 phase is that cyclin D1 can positively regulate the stability of p21 (Coleman et al., 2003), a key cyclin-dependent kinase inhibitor playing a role in the negative regulation of cell cycle progression from the G0/G1 to S phase, by forming complex with p21, which in turn blocks the binding of p21 with C8 α subunit of the 20S proteasome complex. As shown in Fig. 6j, p21 protein level is much higher in $IKK\alpha^{-/-}$ MEFs as compared with that in WT MEFs, which is consistent with the high level of cyclin D1 in $IKK\alpha^{-/-}$ MEFs.

Discussion

Nickel compounds have been well documented as carcinogens in both humans and animals (IARC, 1990). Epidemiological studies have correlated occupational exposure to mixtures of insoluble and soluble nickel compounds with the increased incidence of respiratory, larynx, and nasal cancers (1990; IARC, 1990). Animal studies have shown that insoluble nickel compounds could cause significant tumor formations at the site of administration, whereas the soluble nickel compounds exhibited poor tumor induction (1990; IARC, 1990). Therefore, it is generally thought that insoluble nickel compounds are more potent carcinogens than the soluble. Although many studies have been carried out to explain the different carcinogenic activities of soluble and insoluble nickel compounds, including the studies on the entry and clearance of nickel (Benson et al., 1995), the genotoxicity (Danadevi et al., 2004), epigenetic alterations (Costa et al., 2005; Ke et al., 2006; Salnikow and Costa, 2000), the mechanisms are still not fully understood. In the present study, we demonstrated that soluble nickel compound, $NiCl_2$, can induce a significant cell growth

arrest and cyclin D1 down-regulation, while the insoluble nickel particle, NiS has only a marginal effect.

Cyclin D1 is regulated at both transcription and protein levels. The reduction of cyclin D1 induced by NiCl₂ seems to be caused by proteolysis rather than inhibition of its transcription because the mRNA level of cyclin D1 did not show evident change after the treatment of NiCl₂. Moreover, the pretreatment of MG132, a proteasome inhibitor can reverse the reduction of cyclin D1. Further, we demonstrated that the degradation of cyclin D1 by NiCl₂ requires the IKK α rather than HIF-1 α which are both reported to regulate cyclin D1 (Ghafari et al., 2003; Kwak et al., 2005).

Hypoxia-like response is one major consequence of nickel exposure, which is characterized by the accumulation of HIF-1 α (Costa et al., 2005). HIF-1 α is a transcription factor that was originally found to be activated in cells in response to hypoxia, which forms a heterodimer with HIF-1 β , a constitutively expressed subunit in cells. Previous studies indicated that HIF-1 α may be involved in deregulation of cyclin D1 level (Ghafari et al., 2003). However, in this study, HIF-1 α was not required for the reduction of cyclin D1 by NiCl₂ because exposure of HIF-1 α ^{-/-} MEFs to NiCl₂ still caused similar cyclin D1 reduction.

IKK α , one of the catalytic subunits of IKK complex, primarily mediates the NF- κ B activation. Recently, Kwak et al demonstrated that IKK α can interact with and phosphorylate cyclin D1 at Thr286 residue within the PEST domain (Kwak et al., 2005). IKK α ^{-/-} cells showed exclusive nuclear localization and increased stability of cyclin D1 protein compared with parental MEF cells (Kwak et al., 2005). In this study, we demonstrated that IKK α was also required for the degradation of cyclin D1 by NiCl₂ because the level of cyclin D1 in IKK α ^{-/-} cells did not show any reduction after the exposure to NiCl₂, while the level of cyclin D1 in the parental cells showed a significant reduction. Consistently, the cyclin D1 with T286A point mutation is also resistant to the degradation caused by NiCl₂. However, the growth inhibitory effect of NiCl₂ was only partially rescued by cyclin D1 mutant, which could be explained by either compromising effect of the endogenous cyclin D1 or other possibility that the growth inhibition by NiCl₂ is largely, but not entirely, mediated by cyclin D1 downregulation.

Interestingly, GSK-3 β is also reported to regulate subcellular localization, ubiquitination, and proteolysis of cyclin D1 (Alt et al., 2000; Benzeno and Diehl, 2004; Diehl and Sherr, 1997). The previous studies suggest that GSK-3 β is the physiological kinase mediating cyclin D1 phosphorylation at Thr286 (Alt et al., 2000). In this study, however, we failed to get convincing evidence to show the requirement of GSK3 β for cyclin D1 degradation by NiCl₂, because exposure of cells to NiCl₂ did not induce the de-phosphorylation of GSK3 β , which is required for its activation (Data not shown). In contrast, Akt, the upstream kinase of GSK3 β , has been shown to be activated by the exposure of nickel compounds in our previous study in mouse epidermal C141 cells (Li et al., 2004), which may in turn phosphorylate and inactivate GSK3 β (Cross et al., 1995). In addition, the finding that transfection of dominant negative mutant of GSK3 β can not inhibit the reduction of cyclin D1 by NiCl₂ (Data not shown) also suggests that GSK3 β is not required for the degradation of cyclin D1 in the cell exposed to NiCl₂.

Eukaryotic cell growth is stringently controlled by the well-defined cell cycle consisting of four distinct stages, G1, S, G2, and M. The cell cycle progression is mainly controlled by several key checkpoints, including the G1/S checkpoint, S-phase DNA damage checkpoint and G2/M spindle integrity checkpoint. Previous studies have demonstrated that faulty G1/S control plays a critical role in tumorigenesis. Cyclin D1 is one of the key regulators of G1/S transition (Johnson and Walker, 1999; Kastan and Bartek, 2004; Massague, 2004). Inducible

cyclin D1 forms a complex with CDK4/6 which phosphorylate the retinoblastoma (Rb) tumor suppressor protein, helping to sequester its growth-inhibitory effects and enabling E2F transcription factors to activate genes required for entry into the DNA synthetic phase (S) of the cell division cycle. Accordingly, cyclin D1 is considered as an oncoprotein, and it has been demonstrated that the antisense to cyclin D1 is able to inhibit the growth and tumorigenicity of human colon cancer cells and induce apoptosis in human squamous carcinomas (Arber et al., 1997; Sauter et al., 1999). In this study, we demonstrated that exposure of cells to soluble nickel compound, but not insoluble nickel compound, caused a marked reduction of cyclin D1, which is consistent with their effects on cell cycle alternation. Given the indispensability of cell growth during carcinogenesis, these differences may be at least partially associated with their differential carcinogenic activities.

A critical issue with regard to this study is that the events mediate the different effects of soluble and insoluble nickel compounds on cell growth and cyclin D1 downregulation. Because we treated the cells without any wash, it seems that the persistence of nickel ion in the cells can not be used to explain the difference. Although it is well established that soluble and insoluble nickel compounds exhibit different entry and clearance rate in lungs of animal model, there is no clearance in the cell culture model. Moreover, constant exposure of cells to soluble and insoluble nickel compounds have showed similar profiles of consequences, including signal pathway activations (Huang et al., 2002; Li et al., 2004), target gene expressions (Salnikow et al., 2000; Salnikow et al., 2003a), epigenetic alterations (Ke et al., 2006) and cell transformations (Biedermann and Landolph, 1987; Trott et al., 1995). In our studies, we have also showed that exposure of cells to NiCl₂ and NiS can induce NF- κ B activation and TNF α expression to similar extents (Supplementary Fig. 1), so the difference can not attribute to the possibility of defective function of NiS. In addition, Ni₃S₂, another insoluble nickel compound, exhibited a similar defective effect on cyclin D1 downregulation, but an intact function in HIF-1 α and CAP43 induction as compared with soluble nickel compound. Given that the degradation of cyclin D1 by NiCl₂ requires IKK α , it is possible that soluble and insoluble nickel compounds may have different potency to activate IKK α . However, we failed to see any convincing difference by testing IKK α phosphorylation between the cells exposed to NiCl₂ and NiS. In view of that the degradation of cyclin D1 is an ubiquitination- and proteasome-dependent proteolysis processes, it is possible that soluble and insoluble nickel compounds can differentially regulate this process. This possibility is being tested in our laboratory.

In summary, we demonstrate that soluble, but not insoluble nickel compounds are able to cause a significant cell growth arrest, which results from cyclin D1 degradation in an IKK α -dependent manner as shown in Fig. 6k. These differences may at least in part accounts for their different carcinogenic activities.

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Fig. 1. Inhibition of cell growth of human lung A549 cells by NiCl₂, but not NiS

(a), The cells were treated with various concentrations of nickel compounds as indicated for 24 h and then extracted with SDS-sample buffer. Western blot was carried out with specific

antibodies against HIF-1 α , CAP43 or β -Actin as indicated. **(b and c)**, TNF- α -Luc (b) or NF- κ B-Luc reporter (c) stable transfectants were seeded into each well of 96-well plates, cultured in 10% FBS DMEM medium overnight, and then exposed to NiCl₂ or NiS for 12 h. The cells were then extracted with lysis buffer, and the luciferase activity was measured using the Promega luciferase assay kit. The results were then presented as TNF luciferase activity relative to medium control (relative TNF induction) (b) or NF- κ B transcription activity relative to the control (relative NF- κ B activity) Each bar indicates the mean and standard deviation of triplicate assay wells. The symbol (*) indicates no significant changes between NiCl₂ and NiS ($p > 0.05$). **(d–i)**, 1×10^3 of lung carcinoma A549 cells (d and e), or human bronchial epithelial cell line HCCBE-3 (f and g), or mouse skin epidermal cell line CI41 (h and i) were seeded into each well of 96-well plates, and then exposed to NiCl₂ or NiS. The cells were photographed under microscopy 24 h (h) or 72 h later (d and f), and then extracted by lysis buffer for growth measurement using CellTiter-Glo® Luminescent Cell Viability Assay kit with a luminometer (e, g and i). The results are expressed as inhibition of cell growth which was calculated as described in Material and Methods. Each bar indicates the mean and standard deviation of triplicate assay wells. The symbol (♣) indicates a significant decrease from NiCl₂ group ($p < 0.05$).

Fig. 2. Cell cycle arrest and cyclin D1 degradation induced by NiCl₂, but not NiS, in lung A549 cells

(a), A549 cells were seeded into each well of 6-well plates and cultured in Ham's F-12K containing 10% FBS. After the cell density reached 70–80%, the cells were exposed to 0.25 mM NiCl₂ or 1 µg/cm² NiS for 24 or 36 hrs, and then were fixed and stained with propidium iodide as described in Materials and Methods. Cell cycle distribution was determined by flow cytometry. The data presented in this figure indicating the percentage of G0/G1 and S phases was one representative of three independent experiments. (b and d), The cells were treated with various concentrations of nickel compounds as indicated for 24 h (b), or 0.5 mM of NiCl₂ in the presence or absence of MG132 for 12 h (d), and then extracted with SDS-sample buffer and Western blot was carried out. β-actin was used as a control for protein loading control. (c), The cells were exposed to 0.5 mM NiCl₂ or 2 µg/cm² NiS for 12 h, and then extracted with Trizol reagent for the total RNA isolation. Cyclin D1 was amplified with its specific primers by RT-PCR for 25 cycles. β-actin was used as an internal control.

Fig. 3. Inhibition of MEFs growth by NiCl₂

1×10^3 of MEFs were seeded into each well of 96-well plates, cultured in 10% FBS DMEM overnight, and then exposed to NiCl₂ or NiS for 72 h. The cells were photographed under microscopy (a), and then extracted by lysis buffer for growth measurement using CellTiter-Glo® Luminescent Cell Viability Assay kit with a luminometer (b). The results are expressed as inhibition of cell growth which was calculated as described in Material and Methods. The symbol (♣) indicates a significant different from NiCl₂ group ($p < 0.05$).

Fig. 4. Cell cycle arrest and cyclin D1 degradation induced by NiCl₂ in MEFs

(a), MEFs were seeded into each well of a 6-well plates and cultured in DMEM containing 10% FBS at 37°C overnight. After the cell density reached 70–80%, the cells were exposed to 0.5 mM NiCl₂ for 36 hrs, and then fixed and stained with propidium iodide. The cell cycle distribution was determined by flow cytometry. The data presented in this figure indicating the percentage of G0/G1 and S phases was one representative of three independent experiments. (b and c), MEFs were seeded into each well of 6-well plates, and cultured at 37°C overnight. The cells were treated with 0.5 mM NiCl₂ for 6 or 12 hrs, washed once with ice-cold PBS and then extracted with SDS-sample buffer. The cyclin D1 protein levels in the extracts were analyzed by Western blot (b). The cells were treated with 0.5 mM NiCl₂ for 12 hrs, and then extracted with Trizol reagent for the total RNA isolation. Cyclin D1 was amplified (c).

Fig. 5. HIF-1 α is not involved in cyclin D1 degradation induced by NiCl₂
HIF-1 α ^{+/+} MEFs were seeded into each well of 6-well plates, and cultured in 10% FBS DMEM overnight and exposed to various concentrations of nickel compounds as indicated for 12 hrs, and then subjected to Western blot assay (a). HIF-1 α ^{-/-} MEFs were identified using HIF-1 α antibody (b). The effect of nickel compounds on cyclin D1 protein expression were compared between HIF-1 α ^{+/+} MEFs and HIF-1 α ^{-/-} MEFs by Western blot assay. CDK4 and β -Actin were used as controls (c).

Fig. 6. IKKα is a mediator for cyclin D1 degradation and cell cycle arrest induced by NiCl₂
 (a), A549 cells were treated with 0.5 mM of NiCl₂ in the presence or absence of MG132 for 6 h and 12 h, and then extracted with SDS-sample buffer and Western blot was carried out.
 (b), IKKα^{-/-} MEFs were identified by Western blot. (c), WT MEFs and IKKα^{-/-} MEFs were seeded into each well of 6-well plates and cultured in DMEM containing 10% FBS at 37°C overnight. The cells were exposed to various concentrations of nickel compounds for 12 or 24 hrs, and then extracted with SDS-sample buffer. The cyclin D1 protein levels in the extracts were analyzed by Western blot. CDK4 and β-Actin were used as controls. (d), WT MEFs and IKKα^{-/-} MEFs were seeded into 10-cm dishes and exposed to various concentrations of nickel compounds for 6 or 12 h, and then the nuclear and cytosol parts were extracted as described in “Materials and Methods”. The cyclin D1 protein levels in the extracts were analyzed by Western blot. ATF2 and caspase-3 were used as nuclear and cytosol marks individually. (e–g), 293T cells were transiently transfected with FLAG-cyclin D1 T286A expression vector as described in “Material and Methods”. 24 hrs after the transfection, the cells were treated with 0.5 mM NiCl₂ for 12 hrs and then the FLAG-cyclin D1 T286A mutant and endogenous cyclin D1 protein in the extracts were analyzed by Western blot assay with antibodies against FLAG and cyclin D1 (e). Then the subcellular location of the FLAG-cyclin D1 T286 mutant was detected (f). The growth inhibitory effect of NiCl₂, was compared between the vector control and FLAG-cyclin D1 T286 mutant cells. Each bar indicates the mean and standard deviation of triplicate assay wells. The symbol (♣) indicates a significant compared with vector control cells ($p < 0.05$) (g). (h–j), WT MEFs and IKKα^{-/-} MEFs were seeded into each well of a 6-well plates and cultured in DMEM containing 10% FBS at 37°C overnight. The cells were exposed to various concentrations of nickel compounds as indicated for 24 hrs, and then fixed and stained with propidium iodide. The cell cycle distribution was determined by flow cytometry (h & i). The data presented in this figure indicating the percentage of G0/G1 and S phases was one representative of three

independent experiments, or extracted with SDS sample buffer, and the cell extracts were analyzed by Western blot for p21 protein expression (j). β -Actin was used as protein loading control. (k), The overall scheme of the inhibitory effect on cell growth by soluble, but not insoluble nickel compounds.