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Induction of miR-21-PDCD4 Signaling by Tungsten Carbide-Cobalt Nanoparticles in JB6 Cells Involves ROS-Mediated MAPK Pathways

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ABSTRACT: Tungsten carbide cobalt (WC-Co) nanoparticle composites have wide applications because of their hardness and toughness. WC-Co was classified as "probably carcinogenic" to humans by the International Agency for Research on Cancer (IARC) in 2003. It is believed that the toxicity and carcinogenesis of WC-Co is associated with particle size. Recent studies demonstrated that the tumor suppressor gene programmed cell death 4 (PDCD4) and its upstream regulator miR-21 have been considered as oncogenes for novel cancer prevention or anticancer therapies. The present study examined the effects of WC-Co nanoparticles on miR-21-PDCD4 signaling in a mouse epidermal cell line (JB6 P$^+$). The results showed that (i) exposure of JB6 cells to WC-Co stimulated a increase of miR-21 generation; (ii) WC-Co also caused inhibition of PDCD4, a tumor suppressor protein and downstream target of miR-21, expression in JB6 cells; (iii) inhibition of ERKs with ERK inhibitor U0126 significantly reversed WC-Co induced PDCD4 inhibition, but inhibition of p38 with p38 inhibitor SB203580 did not; and (iv) ROS scavengers, N-acetyl-L-cysteine and catalase, blocked the inhibitory effect of WC-Co on PDCD4 expression, while superoxide dismutase promoted the inhibitory effect. These findings demonstrate that WC-Co nanoparticles induce miR-21 generation, but inhibit PDCD4 production, which may be mediated through ROS, especially endogenous H_2O_2, and ERK pathways. Unraveling the complex mechanisms associated with these events may provide insights into the initiation and progression of WC-Co induced carcinogenesis.

KEY WORDS: JB6 cells, PDCD4, microRNA 21, nanoparticles, tungsten carbide cobalt, nanotoxicology, oncogenicity, cell signal pathways, occupational exposure, occupational respiratory disease

I. INTRODUCTION

Nanotechnology is one of the fastest growing and emerging technologies in the United States and the world. Engineered nanomaterials are used in large amounts in several industries and an increasing demand, including new types of particles, is anticipated in the future.[1] Their physicochemical properties, i.e., the small size and the high surface area–to-volume ratio, are some of their most interesting characteristics, which are not only useful for many applications in medicine, chemistry, and material sciences and physics, but also bring about undesired health effects.[2]

Hard metal or cemented carbide (WC-Co), which consists of tungsten carbide (WC) and metallic cobalt (Co), exhibits a unique combination of hardness and toughness with wide industrial applications. Occupational exposure to hard metal dust is associated with an increased risk of lung cancer.[3] Tungsten heavy alloys are materials that are used broadly for radiation shielding in both medical equipment and oil well drilling industries, as weights and counterbalances, for boring bars and grinding quills, and as tooling for die cast manufacturing. Development of nanosized materials is of emerging technological importance for creation of novel products in different industries. Nanograined WC-Co composites are exceptional surfacing materials applied for hard coatings, decarburization, and metal spraying.[4] WC-Co is employed for cut-

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ting tools, metal forming tools, mining tools, and wear-resistant surfaces, with a wide range of applications, ranging from aerospace and automotive to home appliance products. For these applications, the mechanical properties of interest are hardness, toughness, compressive strength, transverse rupture strength, and wear resistance. The synthesized nanopowders provide better sinterability and mechanical properties with a homogeneous microstructure. Nano–WC-Co coating is essential for cutting tools and dies providing high hardness, toughness, and high wear- and erosion-resistant properties.[5] Epidemiological studies have identified an increased risk of lung cancer among workers exposed to hard metal dusts.[6,7] Notably, De Boeck et al.[3] reported that occupational exposure to hard metal dust, consisting of WC and Co particles, is associated with an increased risk of lung cancer. Based on sufficient evidence obtained from laboratory studies and epidemiological studies in humans, the International Agency for Research on Cancer (IARC) has classified tungsten carbide cobalt as "probably carcinogenic" to humans.[8]

Enhanced toxic effects of nanosized WC-Co compared to fine-sized WC-Co have been observed.[9] Previous studies have shown that WC-Co nanoparticles induced high levels of reactive oxygen species (ROS) and stimulated oncogenes activation, including MAPKs, NF-κB, and AP-1.[10] AP-1 activation may contribute to the toxicity and carcinogenesis of WC-Co particles.[10] However, signaling pathway in the activation of AP-1 by WC-Co remains to be investigated. Research on the elucidation of the mode of action of these materials is of high relevance for occupational health. Recently, the novel tumor suppressor gene, programmed cell death 4 (PDCD4), has been identified as negative regulator of AP-1.[11] But the roles of PDCD4 in the AP-1 activation induced by WC-Co need to be investigated.

PDCD4 was first identified as a tumor suppressor gene and act as neoplasmic transformation inhibitor in JB6 cells by Cmarik et al.[12] PDCD4 was originally isolated from a human glioma library and is homologous to the mouse PDCD4 (MA-3/TIS/A7-1) gene.[13] It has been shown to inhibit the activation of AP-1–dependent transcription, skin tumorigenesis, and tumor progression in transgenic mice.[13] Overexpression of PDCD4 inhibits TPA-induced transformation in JB6 cells[14] and tumor phenotype of transformed cells.[15] Knockout of PDCD4 in mice results in induction of lymphomas with frequent metastasis[16] and an increase in DMBA/TPA-induced skin papilloma formation and carcinoma incidence.[17] These findings suggest that PDCD4 suppresses carcinogenesis at both promotion and progression stages. Early studies indicated that PDCD4 is a target of micro-RNA-21 (miR-21) in colon cancer[18] and human breast cancer cells.[19] Micro-RNAs (miRNAs) are small non–protein coding RNAs approximately 19–25 nucleotides in length, which negatively regulate gene expression by binding to specific sites on the 3' untranslated region (UTR) of target mRNAs and causing translational repression. Alteration of miRNA expression caused by exposure to different carcinogens has been well reported. miR-21 has emerged from many profiling experiments as a miRNA whose expression is dysregulated in cancer.[20,21] PDCD4 has emerged as a major, functionally significant target of miR-21.[11,22,23] PDCD4 and miR-21 have been considered as potential targets for novel cancer prevention or anticancer therapies.[13,22] The observed inverse correlation between PDCD4 and miR-21 expression may be a useful prognostic marker.[22,24]

A few data on adverse effects of nanosized WC-Co particles have been reported. To address potential carcinogenic properties of nanosized WC-Co particles, we evaluated their effects on PDCD4 and miR-21 expression in JB6 cells. We observed that *in vitro* exposure of JB6 cells to WC-Co nanoparticles caused inhibition of PDCD4 expression and induction of miR-21 generation. ROS, especially H_2O_2 as well as ERKs pathway, are involved in these responses.

II. METHODS AND MATERIALS

A. Reagents

Eagle's minimal essential medium (MEM) and phosphate-buffered saline (PBS) were purchased from Whittaker Biosciences (Walkersville, Maryland). Fetal bovine serum (FBS), gentamicin, and L-glutamine were from Life Technologies, Inc. (Gaithersburg, Maryland). Rabbit antimouse antibodies for PDCD4 phospho-ERKs, and phospho-p38 were from Cell Signaling Technology, Inc. (Boston, Massachusetts). Rabbit antimouse antibody for β-Tubulin, and goat antirabbit IgG-HRP secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, California). ERK inhibitor U0126 (MEK1 inhibitor), p38 inhibitor SB203580 were from Cell Signaling Technology, Inc. (Boston, Massachusetts). Reactive oxygen species (ROS) scavenger superoxide dismutase (SOD), catalase (CAT), N-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, Missouri).

B. Preparation of Particles

Nanosized WC-Co (average grain size 80 nm, 99.9% pure, molecularly mixed at the ratio of 85:15, agglomerated powder) were obtained from Inframat Advanced Materials LLC (Farmington, Connecticut). Stock solutions of WC-Co nanoparticles were prepared by sonification on ice using a Branson Sonifier 450 (Branson Ultrasonics Corp., Danbury, Connecticut) in sterile PBS (10 mg/ml) for 30 s, then kept on ice for 15 s and sonicated again for a total of 3 min at a power of 400 W. All samples were prepared under sterile conditions.

C. Cell Culture

The JB6 P^{-} mouse epidermal cell line was cultured in Eagle's MEM containing 5% FBS, 2 mM L-glutamine, and 50 mg/ml gentamicin. The cells were grown at 37°C in a 5% CO_2 atmosphere.

D. Western Blot Analysis

Immunoblotting for expression of PDCD4 and MAPKs was carried out as described by the protocol of manufactures using specific antibodies against each protein. β-tubulin or non-phospho-specific antibodies against ERKs, JNKs, and p38 kinase proteins provided in each assay kit were used to normalize the phosphorylation assay using the same transferred membrane blot.

E. Quantitative Real-Time Polymerase-Chain Reaction (qRT-PCR)

Total RNA was isolated and purified using the miRNA vana isolation kit (Ambion, Austin, Texas) according to manufacturer's protocol. RNA concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technology Inc., Wilmington, Delaware). Each sample was analyzed in duplicate. Relative miR-21 levels were determined by TaqMan real-time polymerase chain reaction (PCR). The reverse transcription PCR reaction was performed at the following conditions: 16°C for 30 min; 42°C for 30 min; 85°C for 5 min, and then hold at 4°C. After the RT-PCR reaction, the cDNA products were used for PCR reaction along with TaqMan primer. The PCR reaction was carried out at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Values obtained from untreated cells were set to 1.

F. EFFECT OF INHIBITORS/ANTIOXIDANTS ON PDCD4 ALTERATION INDUCED BY WC-CO.

JB6 P^{-} cells were pretreated with ERK inhibitor U0126 (20 μM), p38 inhibitor SB203580 (20 μM), catalase (10,000 U/ml), N-acetyl-cysteine (NAC, 10 mM), or superoxide dismutase (SOD, 1000 U/ml) for 1 h and then exposed to WC-Co in the presence of the same reagents. PDCD4 or miR-21 expression was assayed as described earlier.

G. Statistical Analysis

Data presented are the means ± standard errors of *n* experiments as noted in the figure captions. Outcome variables were analyzed using analysis of variance (ANOVA) and Student's *t*-tests. Significance was set at $p < 0.05$.

III. RESULTS

A. Surface area and size distribution of WC-Co nanoparticles

To measure the surface area and size distribution of WC-Co nanoparticles, a Gemini 2360 surface area analyzer and SEM were used, respectively. The average surface area of WC-Co nanoparticles was 2.73 m^2/g. The average size distributions of WC-Co nanosized particles were 95.53 nm, as described previously.[10]

B. WC-Co nanoparticles inhibited PDCD4 expression

It has been reported that WC-Co nanoparticles stimulate MAPK-AP-1 pathway[10] and that PDCD4 was involved in AP-1–dependent transcription.[22] We assumed that PDCD4 is involved in the WC-Co nanoparticles–induced cellular response. To verify this, 3×10^5 JB6 cells were exposed to various concentrations of WC-Co nanoparticles and the PDCD4 expression was tested. The results indicated that WC-Co caused a dose-dependent decrease of PDCD4 expression [Figs. 1(a) and 1(b)] and a dose-dependent increase in phosphorylated ERKs [Figs. 1(a) and 1(c)]. At the concentration range of 50–150 μg/cm^2, WC-Co induced a significant decrease of PDCD4 expression compared to untreated control.

Time-course study revealed that inhibition of PDCD4 was first observed after 0.5 h of incubation (200 μg/cm^2); and thereafter, the expression of PDCD4 decreased to a maximum at 2 h and lasted until 6 h. Further incubations of cells (12 h) with WC-Co resulted in a poor response [Figs. 2(a)

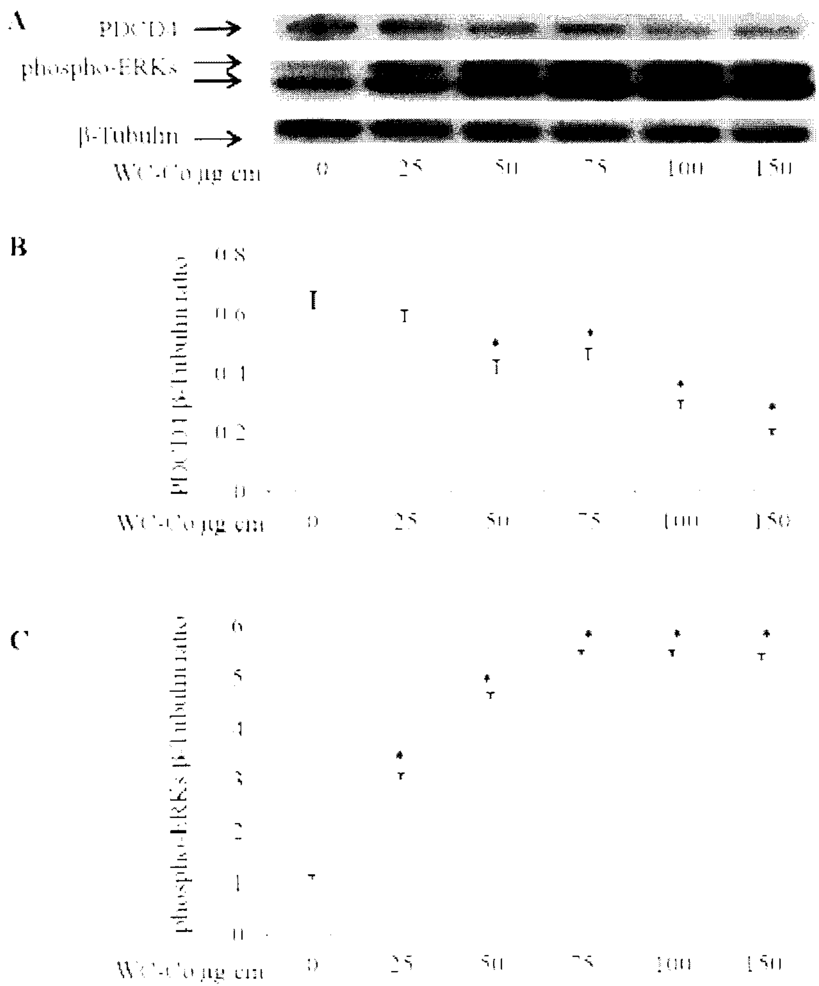

FIGURE 1. Effects of nanosized WC-Co particles on PDCD4 and phospho-ERKs expression in JB6 P$^+$ cells. JB6 cells were cultured in MEM containing 5% FBS in six-well (35 mm diameter) plates until 80% confluent and then cultured in MEM containing 0.1% FBS for 24 h. The cells were then exposed to various concentrations of nanosized WC-Co for 2 h. The cells were lysed. PDCD4, phosphated ERK, and β-Tubulin expressions were analyzed using the same transferred membrane blot. (a) The representative Western blotting image. (b) The quantitative analysis of PDCD4 protein normalized to β-tubulin levels. (c) The quantitative analysis of phospho-ERKs protein normalized to β-tubulin levels. The results are mean ± SEM, a representative of four different experiments; *$p < 0.05$ versus untreated cells.

and 2(b)]. Phosphorylation of ERKs increased in a time-dependent manner as expected [Figs. 2(a) and 2(c)]. These results suggest that PDCD4 expression was inhibited while ERKs were activated by WC-Co in JB6 cells.

C. Effect of WC-Co nanoparticles on miR-21 expression

Since PDCD4 has been demonstrated to be a major, functionally significant downstream target, as well as a upstream regulator of miR-21,[23] PDCD4 and miR-21 have been considered as potential targets for novel cancer prevention or anticancer therapies.[13,22,24] We proposed that miR-21 expression might increase after exposure of the cells to WC-Co. To test this hypothesis, the cells were exposed to various concentrations of nanosized WC-Co for 0.5, 1, 2, and 4 h. Total RNA were purified from the respective cell pellets and analyzed by qRT-PCR for the expression of miR-21. Values obtained from untreated cells were set to 1. The results showed that at the concentration of 25 and 50 μg/cm² of WC-Co, the miR-21 ex-

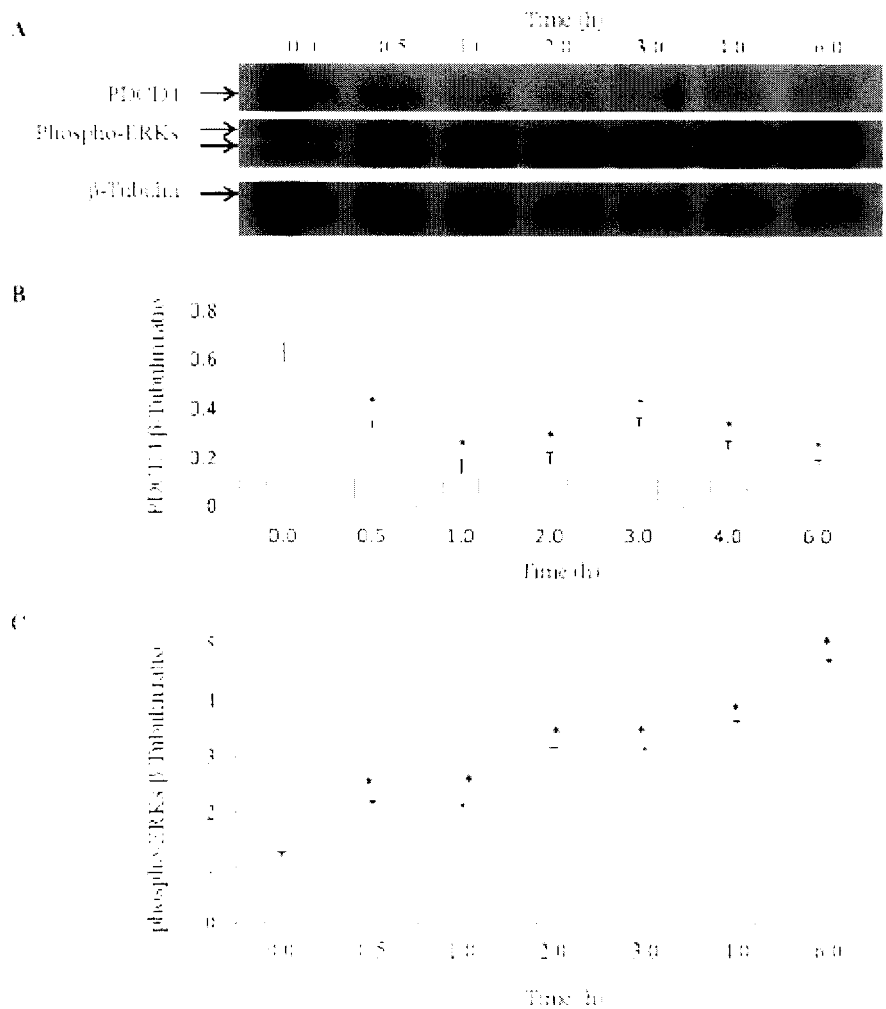

FIGURE 2. Time-course study on PDCD4 and phospho-ERKs expression in JB6 cells treated with WC-Co nanoparticles. JB6 cells were cultured in MEM medium containing 5% FBS in six-well (35 mm diameter) plates until 80% confluent and starved in MEM media containing 0.1% FBS for 24 h. The cells were then exposed to 200 μg/cm² WC-Co nanoparticles for different times as indicated. The cells were lysed, and PDCD4, phospho-ERKs, and β-Tubulin expressions were analyzed by Western blot. (a) The representative Western blotting image. (b) The quantitative analysis of PDCD4 protein normalized to β-tubulin levels. (c) The quantitative analysis of phospho-ERKs protein normalized to β-tubulin levels. The results are mean ± SEM, a representative of three different experiments; *$p < 0.05$ versus untreated cells.

pression level was significantly increased in JB6 cells at 1, 2, and 4 h postexposure (Fig. 3). These results indicate that WC-Co induced miR-21 expression in JB6 cells.

D. Effects of ERKs or p38 inhibitors on WC-Co-induced PDCD4 alteration

Previous studies from our laboratory have shown that WC-Co causes AP-1 activation *in vitro* using cell culture systems and *in vivo* using transgenic mice, and that ERKs and p38 are involved in the process of WC-Co–induced AP-1 activation.[10] It is known that AP-1 is a key member of miR-21–PDCD4 signaling cascade.[11,22,23] It is possible that MAPKs-AP-1 may be involved in WC-Co–induced PDCD4 and miR-21 alteration. Since WC-Co stimulates phosphorylation of ERKs and p38 kinase, we examined the effects of ERKs or p38 inhibitors on WC-Co–induced alteration of PDCD4 expression in JB6 cells treated with WC-Co. Cells were pretreated for 1 h with ERK inhibitor U0126 (20 μM) or p38 inhibitor SB203580 (20 μM), and then exposed to WC-Co in the presence of the inhibitors for 2 h. PDCD4 expression was analyzed by Western blot. The results show that inhibition of ERKs with ERKs inhibitor, U0126, blocked WC-Co–induced inhibition of PDCD4, while inhibition of p38 with p38 inhibitor, SB203580, did not (Fig. 4). These results suggest that ERKs, but not p38, might mediate WC-Co–induced PDCD4 inhibition in JB6 cells.

E. Effects of antioxidant reagents on WC-Co-induced PDCD4 inhibition

It has been suggested that ROS plays a critical role in WC-Co–induced MAPKs and AP-1 activation and that ROS scavenger N-acetylcysteine (NAC) blocked WC-Co–induced AP-1 activation and ERKs, p38 phosphorylation.[10] We hypothesized that ROS might also be involved in WC-Co–induced PDCD4 inhibition. To examine this hypothesis, the effects of ROS scavenger, catalase (CAT), NAC, and superoxide dismutase (SOD) on WC-Co–induced alteration of PDCD4 were

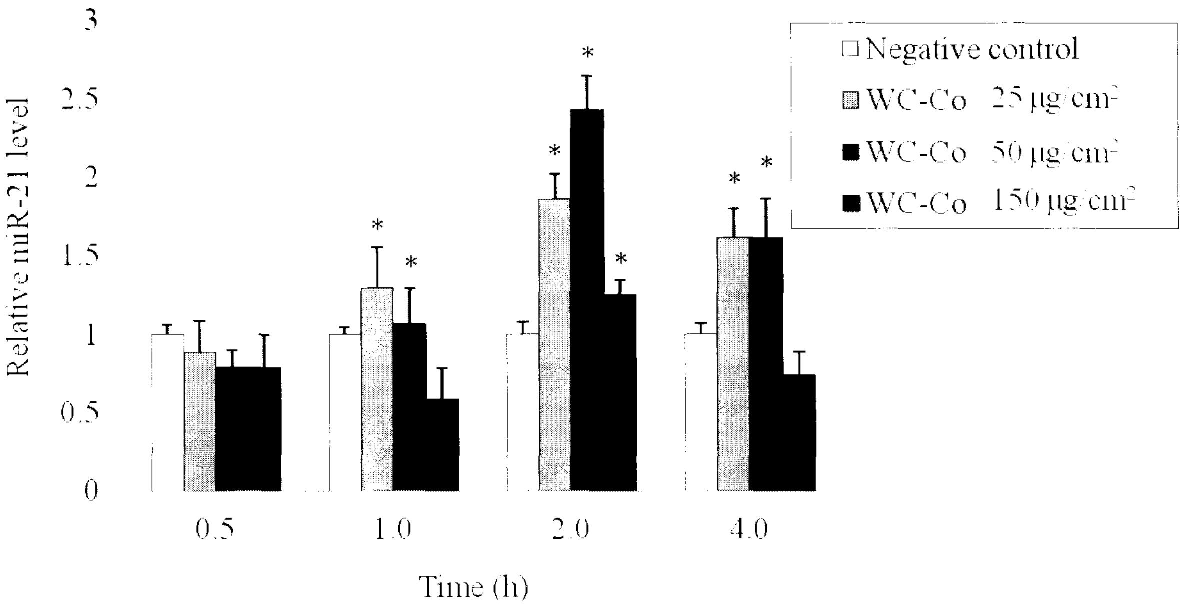

FIGURE 3. Effect of miR-21 expression in JB6 cells postexposure to WC-Co. JB6 cells were exposed to various concentrations of WC-Co for the times indicated. Total RNA were purified. To check miRNA levels, reverse transcription (RT) was performed using TaqMan microRNA reverse transcriptase kit and the RT primers for miR-21 and control U6 (Applied Biosystems). Taqman real-time PCR reaction was performed using Fast TaqMan Universal Master Mix and the TaqMan probe and forward primer for miR-21 or U6. All primers were purchased from Applied Biosystems. All reactions were performed according to manufacturer's protocols. Normalizations for miRNA real-time PCR were performed using the Ct of U6. The results are mean ± SEM, a representative of three experiments; *$p < 0.05$ versus untreated control cells.

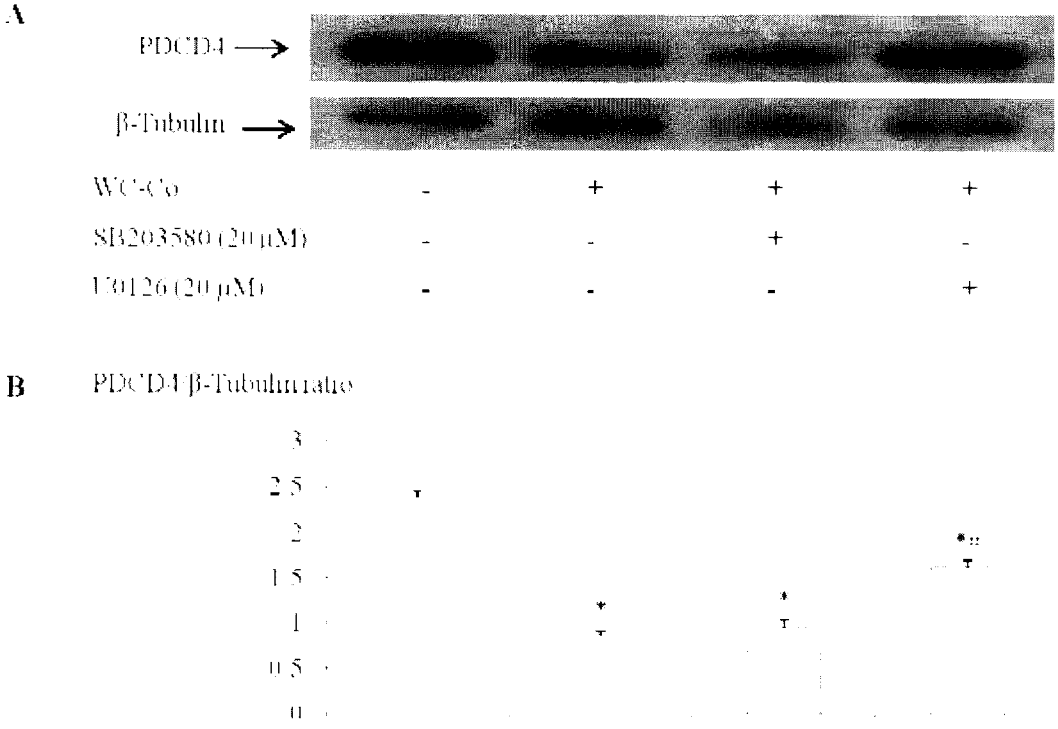

FIGURE 4. Effects of ERK or p38 inhibitors on WC-Co–induced inhibitory expression of PDCD4 in JB6 cells. The cells were pretreated with ERK inhibitor U0126 (20 μM) or p38 inhibitor SB203580 (20 μM) for 1 h, then exposed to 150 μg/cm^2 of nanosized WC-Co in the presence of the same reagents for 2 h. The cells were lysed. The PDCD4 as well as β-Tubulin expressions were analyzed using the same transferred membrane blot. (a) The representative Western blotting image. (b) The quantitative analysis of PDCD4 protein normalized to β-tubulin levels. The results are mean ± SEM, a representative experiment with JB6 P+ cells from three different experiments; *$p < 0.05$ versus untreated negative control; #: $p < 0.05$ versus positive control.

investigated. JB6 cells were pretreated with ROS scavengers, CAT, NAC, or SOD for 1 h, and then exposed to WC-Co in the presence of the same reagents for 2 h. Following these exposures, PDCD4 expression or phosphorylated ERKs and p38 were tested in JB6 cells (Fig. 5).

The results showed that pre-exposure of the cells to NAC, a thiol-containing antioxidant that was shown to reduce the ROS and protect against the toxic effects of ROS,[25] blocked the inhibitory effect of PDCD4 expression induced by WC-Co. CAT, a H_2O_2-scavenging enzyme, also reversed inhibitory action of WC-Co on PDCD4 expression. Whereas, pre-exposure of the cells to SOD, an O_2^- scavenger that generates H_2O_2, resulted in synergistic inhibition of PDCD4 production. These results indicated that ROS, especially endogenous H_2O_2, might be the mediator for stimulation of MAPKs pathways leading to PDCD4 inhibition.

IV. DISCUSSION

Occupational exposure of workers to the WC-Co particle mixture has been shown to be carcinogenic.[7] The International Agency for Research on Cancer (IARC) has classified WC-Co and its compounds as possibly carcinogenic to humans.[26] However, the molecular mechanisms involved in hard metal–induced carcinogenesis are unclear. We hypothesized that activation of nuclear transcription factors induced by WC-Co is a primary event in the initiation of signal transduction cascades at the cellular level in exposed individuals. We previously observed that exposure of mouse epidermal cells (JB6 P$^+$) induced ROS mediated AP-1 transactivation.[10]

In this study, we observed that treatment of JB6 P$^+$ with WC-Co nanoparticles caused reduction of PDCD4 expression and generation of ROS,

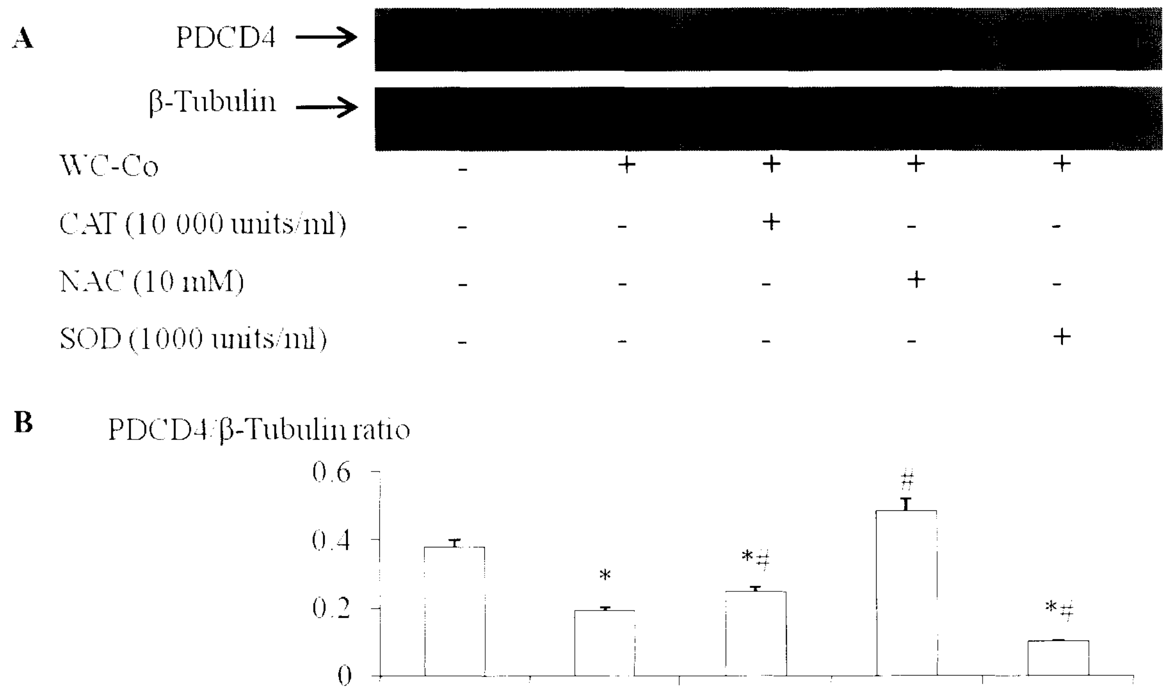

FIGURE 5. Effects of ROS scavengers on PDCD4 expression in JB6 cells treated with WC-Co nanoparticles. The cells were pretreated with ROS scavenger catalase (CAT) (10,000 units/ml), N-acetyl-L-cysteine (NAC) (10 mM), superoxide dismutase (SOD) (1000 units/ml) for 1 h, then exposed to 150 μg/cm^2 of WC-Co nanoparticles in the presence of the same reagents for 2 h of culturing time. The PDCD4 expression was analyzed using the same transferred membrane blot. (a) The representative Western blotting image. (b) The quantitative analysis of PDCD4 protein normalized to β-tubulin levels. The results are mean ± SEM of three assays from three different experiments; *$p < 0.05$ versus untreated cells; #: $p < 0.05$ versus positive control (treated with only WC-Co particles).

along with stimulation of phosphorylated MAPKs and miR-21 production. Nanosized WC-Co induced a threefold decrease in PDCD4 expression and a twofold increase in miR-21 generation in JB6 cells. Inhibition of ERKs with ERK inhibitor, U0126, significantly reversed WC-Co–induced PDCD4 inhibition, but inhibition of p38 with p38 inhibitor SB203580 did not. Pre-exposure of the cells to NAC or CAT reversed inhibitory action of WC-Co on PDCD4 expression, while pretreatment of the cells to SOD resulted in synergistic inhibition of PDCD4 expression. Taken together, these results clearly demonstrated that WC-Co nanoparticles stimulated proto-oncogenes miR-21-PDCD4 signaling and that ROS, especially H_2O_2, plays a critical role in these effects.

PDCD4 has been known to play an important role in antitumor promotion.[13] PDCD4 and its upstream regulator miR-21 have been considered as potential targets for novel cancer prevention or anticancer therapies.[13] Jansen et al. reported that transgenic mice overexpressing PDCD4 in the epidermis exhibited significant reductions in tumor promotion,[27] whereas Yang et al. noted that an elevated expression of PDCD4 inhibited tumor promotion of JB6 P^+ cells induced by TPA and tumorigenic JB6 RT101 (Tx) cells.[14,15] PDCD4 functions to inhibit AP-1–dependent transcription that is believed to be critical for tumorigenic phenotype.[14,28] PDCD4 is a tumor suppresser protein and was identified in the JB6 mouse epidermal clonal genetic variant cell system that is preferentially expressed in tumor promoter–resistant cells (JB6P^- cells), but suppressed in promotion-sensitive cells (JB6 P^+ cells).[12] Overexpression of sense PDCD4 in P^+ cells is sufficient to inhibit tumor promoter TPA-induced neoplastic transformation and produces a transformation-resistant phenotype, possibly through inhibiting AP-1–dependent transcriptional activation.[14,15] We have demonstrated that nanosized WC-Co activates AP-1 and NF-κB more efficiently in mouse JB6 P^+ cells as compared to fine WC-Co.[10] In this study, we observed that PDCD4 expression was inhibited in JB6 cells postexposed to WC-Co. These results indicate that similar to arsenite,[28] WC-Co may exert its potential carcinogenesis through PDCD4.

It has been reported that the interaction of miR-21 and PDCD4 is highly conserved among animals.[23] The miR-21s from human, chimpanzee, monkey, mouse, rat, chicken, and pig are 100% identical and 95% identical to that of zebra fish, puffer fish, and cow.[29] Both human and mouse PDCD4 genes are predicted to be a target of miR-21 by four widely used computational methods, Miranda, TargetScan, PicTar, and RNA22.[23] It appears that further investigation on the molecular regulation of PDCD4 is needed to understand the mechanisms underlying WC-Co–associated carcinogenesis. The signal transduction pathways leading to miR-21–PDCD4 signaling have been reported early.[30] It is believed that stress-related signals such as UV light or reactive oxygen species induce the activation of MAPK pathways (ERKs, JNKs, and p38) leading to transactivation of AP-1 activity. miR-21 is a downstream target of AP-1 transcription factor, as well as an upstream regulator.[11] In this study, the possible role of the MAPK family, including p38 kinase and ERKs, in WC-Co–induced alteration of miR21–PDCD4 has been investigated. We found that WC-Co stimulated phosphorylated ERKs and p38 kinase. Pretreatment of cells with the ERK inhibitor, U0126, significantly blocked WC-Co–induced PDCD4 inhibition, but inhibition of p38 with p38 inhibitor, SB203580, did not. Thus, these results suggest that WC-Co–induced inhibition of PDCD4 might be through ERKs, but not p38 pathways.

Oxidative stress is involved in WC-Co–induced toxicity and AP-1 activation.[10] Previous studies have indicated that ROS are associated not only with initiation but also with promotion and progression in the multistage carcinogenesis mode.[31] It has been reported that oxidative stress induces activation of transcription factors and the expression of several oncogenes including AP-1, NF-κB, c-myc, and c-fos, which enhance cell proliferation.[32] Reducing oxidative stress suppresses the proliferation of tumor cells.[33] The results from the present study show that WC-Co–induced alteration of PDCD4 expression involves ROS-mediated reactions. A major role of H_2O_2 in WC-Co–induced PDCD4 suppression is supported by the following observations: (i) NAC, a thiol-containing antioxidant, completely blocked WC-Co–induced inhibitory effect on PDCD4 expression, and (ii) catalase, whose function is to remove H_2O_2, also impeded WC-Co–induced inhibition of PDCD4; (iii) SOD, which converts $O_2^{\cdot}$ to H_2O_2, enhanced inhibitory effect. These results clearly suggest that H_2O_2 plays a critical role in the inhibition of PDCD4 expression.

In conclusion, the results obtained herein clearly demonstrate that WC-Co inhibits PDCD4, a tumor suppressor gene, expression and induces miR-21, a proto-oncogene, over expression through ROS and ERKs signaling. These studies provide new and important clues regarding molecular mechanisms that may be involved in WC-Co–induced carcinogenesis. Therefore, elucidating the mechanisms involved in WC-Co–induced carcinogenesis in parallel with the manipulation of target signaling could provide insights for the understanding and possible prevention of WC-Co–induced carcinogenesis.

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