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Reactive oxygen species mediate Cr(VI)-induced carcinogenesis through PI3K/AKT-dependent activation of GSK-3 β / β -catenin signaling

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Abstract

Cr(VI) compounds are known human carcinogens that primarily target the lungs. Cr(VI) produces reactive oxygen species (ROS), but the exact effects of ROS on the signaling molecules involved in Cr(VI)-induced carcinogenesis have not been extensively studied. Chronic exposure of human bronchial epithelial cells to Cr(VI) at nanomolar concentrations (10 - 100 nM) for 3 months not only induced cell transformation, but also increased the potential of these cells to invade and migrate. Injection of Cr(VI)-stimulated cells into nude mice resulted in the formation of tumors. Chronic exposure to Cr(VI) increased levels of intracellular ROS and antiapoptotic proteins. Transfection with catalase or superoxide dismutase (SOD) prevented Cr(VI)-mediated increases in colony formation, cell invasion, migration, and xenograft tumors. While chronic Cr(VI) exposure led to activation of signaling cascades involving PI3K/AKT/GSK-3 β / β -catenin and PI3K/AKT/mTOR, transfection with catalase or SOD markedly inhibited Cr(VI)-mediated activation of these signaling proteins. Inhibitors specific for AKT or β -catenin almost completely suppressed the Cr(VI)-mediated increase in total and active β -catenin proteins and colony formation. In particular, Cr(VI) suppressed autophagy of epithelial cells under nutrition deprivation. Furthermore, there was a marked induction of AKT, GSK-3 β , β -catenin, mTOR, and carcinogenic markers in tumor tissues formed in mice after injection with Cr(VI)-stimulated cells. Collectively, our findings suggest that ROS is a key mediator of Cr(VI)-induced carcinogenesis through the activation of PI3K/AKT-dependent GSK-3 β / β -catenin signaling and the promotion of cell survival mechanisms via the inhibition of apoptosis and autophagy.

Keywords

Cr(VI); Cell transformation; Carcinogenesis; Reactive oxygen species (ROS); β -catenin

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Introduction

Chromate (Cr(VI)) compounds are widely used in industry for plating, welding, and pigment production (Cohen et al., 1993; Costa, 1997). Occupational exposure to Cr(VI) is a well-established cause of lung cancer (De Flora et al., 1990; Freeman et al., 1997; Hayes, 1988; Langard, 1990; Singh et al., 1998). Therefore, Cr(VI) is included in the list of class I carcinogens (IARC, 1990).

Reactive oxygen species (ROS) are reactive short-lived oxygen-containing species such as hydroxyl radical ($\cdot\text{OH}$), superoxide anion ($\text{O}_2^{\cdot-}$), and hydrogen peroxide (H_2O_2). Intracellular reduction of hexavalent chromium (Cr(VI)) is associated with the production of ROS (Shi and Dalal, 1994; Shi et al., 1992; Shi and Dalal, 1989; Shi and Dalal, 1990), which are known to cause oxidative damage such as DNA strand breaks, base modification, and lipid peroxidation (Ding and Shi, 2002; Hodges et al., 2001; Shi et al., 1999; Stohs et al., 2000; Xu et al., 1992; Ye et al., 1995). Thus, it is generally believed that ROS induced by Cr(VI) could contribute to carcinogenesis (Shi et al., 1994; Shi et al., 1998; Shi and Dalal, 1989; Shi and Dalal, 1990; Wang et al., 2000; Ye et al., 1995). However, there has not been any critical evidence demonstrating the involvement of ROS in Cr(VI)-induced carcinogenesis. Recently, our group reported that chronic exposure of lung epithelial cells to Cr(VI) induced cell transformation through a NOX-dependent increase in ROS production (Wang et al., 2011). We have extended our analysis to study how ROS signaling mediates the transformation of normal cells. Our primary goal is to understand the cellular mechanisms by which ROS regulates the signaling pathways involved in Cr(VI)-induced carcinogenesis.

Cell migration and invasion are indicative of malignant tumor growth, which involves cell proliferation, adhesion, proteolytic degradation of tissue, and angiogenesis (Fan et al., 2006). Matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) are also involved in this process (Gordon et al., 2010; Jiang et al., 2001). Most of all, angiogenesis is a key event in cancer development and tumor progression. VEGF is an important mediator of angiogenesis (Miyoshi and Ohshima, 2001). The expression of VEGF is induced by hypoxia-inducible transcription factor-1 α (HIF-1 α under hypoxic condition in tumors (Bedogni et al., 2005). ROS has been shown to modulate these effects through induction of transcription factors or genes involved in angiogenesis and carcinogenesis. However, high ROS levels suppress angiogenesis and carcinogenesis (Nishikawa, 2008).

The β -catenin signaling pathway plays a critical role in cell transformation and carcinogenesis (Behrens and Lustig, 2004; Behrens, 2005; Klaus and Birchmeier, 2008). β -catenin can regulate the transcription of proto-oncogenes such as *c-myc*, *cyclin D1*, and *ABCB1* (Chakraborty et al., 2010), *VEGF* (Zhang et al., 2001), *COX-2* (Howe et al., 2001), and *MMPs* (Marchenko et al., 2002). Furthermore, it has been reported that β -catenin stabilizes telomerase in human cancer, which is a hallmark of tumorigenesis, through enhanced *Tert* expression (Katrin Hoffmeyer, 2012). In response to Wnt signals, dephosphorylated β -catenin accumulates in the cytoplasm and is transported to the nucleus. Once in the nucleus, β -catenin regulates numerous target genes. Phosphorylated β -catenin becomes multi-ubiquitinated and is subsequently degraded in proteasomes (Lustig and Behrens, 2003). In addition, the serine/threonine kinase GSK-3 β is constitutively active in unstimulated cells (Cohen and Frame, 2001). GSK-3 is a downstream effector of the PI3K/AKT pathway, and its activity is inhibited by AKT-mediated phosphorylation at residue Ser 9 (Cross et al., 1995). GSK-3 β also tightly regulates β -catenin signaling; phosphorylation of β -catenin by GSK-3 β leads to ubiquitin-mediated degradation of β -catenin in proteasomes (MacDonald et al., 2009). Because β -catenin signaling is regulated by ROS in various types of cells (Heo and Lee, 2011; Ladelfa et al., 2011), it is likely that Cr(VI) exerts its

transformative and carcinogenic effects by increasing cellular ROS levels and activating β -catenin signaling.

Autophagy is a cellular defense process in which cytosolic components, organelles, and invading bacteria are transported by autophagosomes to lysosomes for degradation (Dice, 2007; Levine and Klionsky, 2004; Mizushima, 2007; Muller et al., 2000). Recent work has highlighted the relationship between autophagy and tumorigenesis. For example, autophagy supports cell survival in hypoxic tumor regions (Degenhardt et al., 2006; Karantza-Wadsworth et al., 2007). Paradoxically, PI3K and mTOR, which are negative regulators of autophagy, are highly expressed in human tumors (Jin and White, 2007; Jin and White, 2008; Levine and Kroemer, 2008; Mathew et al., 2007). In addition, it has been reported that autophagy suppresses tumorigenesis through the elimination of p62 (Mathew et al., 2009).

Although Cr(VI) is a well-established carcinogen, limited information is available on the role of ROS in Cr(VI)-induced carcinogenesis. Furthermore, the mechanisms by which ROS regulate Cr(VI)-mediated carcinogenic signaling is unclear. In this study, we examined the transformative and carcinogenic effects of Cr(VI) using a human bronchial epithelial cell line, BEAS-2B, and an animal xenograft model. We also investigated the roles of ROS in Cr(VI)-induced carcinogenesis and the signal transduction pathways involved.

Materials and methods

Chemicals and supplies

Unless specified otherwise, all chemicals and laboratory equipment were purchased from Sigma Chemical Co. (St. Louis, MO) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ), respectively. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), gentamicin, and L-glutamine were purchased from Gibco Co. (Gibco BRL, NY). The PI3 kinase inhibitor LY294002 was obtained from Cell Signaling (Beverly, MA). Inhibitors specific for GSK-3 (SB216763) and β -catenin (FH535) were purchased from Calbiochem (San Diego, CA).

Cell culture and treatment

The human bronchial epithelial cell line BEAS-2B was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin. The cells were exposed continuously to $\text{Na}_2\text{Cr}_2\text{O}_7$ (0-100 nM) in the media. Cells were sub-cultured every week for 3 months and before processing for experiments.

Plasmids and transfection

CAT-Myc-DDK- and SOD1-Myc-DDK-tagged plasmids were purchased from Origene (Rockville, MD). The SOD2-EGFP-tagged plasmid was obtained from Addgene (Cambridge, MA). Transfections were performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, BEAS-2B cells were seeded in 6-well culture plates and transfected with 4 μg plasmid at approximately 50% confluency. Expression of CAT, SOD1, and SOD2 protein was measured by immunoblotting, and stable cell lines were maintained using G418.

Anchorage-independent colony growth assays

Soft agar colony formation assay was performed as described previously (Son et al., 2012). Briefly, 3 ml of 0.5% agar in DMEM supplemented with 10% FBS was spread onto each well of a 6-well culture plate. A suspension (1 ml) containing Cr(VI)-exposed BEAS-2B cells (1×10^4) was mixed with 2 ml of 0.5% agar-DMEM and layered on the top of the

0.5% agar layer. The plates were incubated at 37°C in 5% CO₂ for 2 months, and colonies larger than 50 µm in diameter were counted under a light microscope.

Clonal assay

The clonal assay was performed as described elsewhere (Plaisant et al., 2011). Cr(VI)-exposed cells were plated in 6-well plates at a density of 1×10^3 cells/well and incubated for 7 days with two media changes. The cells were fixed with 2% formalin for 10 min and stained with 0.5% crystal violet for 5 min prior to observation by light microscopy.

Cell migration assay

Cell migration was measured using transwell chambers (8-µm pore size, BD Biosciences) according to methods described elsewhere (Son et al., 2012). The top chambers of the transwells were loaded with 0.2 ml cells (5×10^5 cells) and the bottom chambers contained 0.7 ml complement medium. After incubation at 37°C and 5% CO₂ for 12 h, the migrated cells were fixed, stained with hematoxylin solution, and counted under a microscope.

Cell invasion assay

The cell invasion assay was performed according to methods described previously (Qian et al., 2003). Cr(VI)-stimulated BEAS-2B cells (5×10^5 cells) were loaded into 24-well invasion chambers (BD Biosciences) that were pre-coated with 100 µl Matrigel solution. Medium containing 10% fetal calf serum was added to the plates (0.7 ml/well). The Matrigel invasion chambers were incubated for 3 days, and the invaded cells were stained with hematoxylin and observed by microscopy.

Western blot analyses

Cell lysates were prepared in RIPA buffer (20 mM Tris-Cl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM EGTA, 1% NP-40, 1% sodium-deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin and 1 mM PMSF). Equal amounts of protein (30 µg/sample) were separated by the NuPAGE Bis-Tris electrophoresis system (Invitrogen, Carlsbad, CA) and blotted onto nitrocellulose membrane (Whatman, Dassel, Germany). Blots were probed with primary and secondary antibodies before exposure to Hyperfilm (Amersham Pharmacia Biotech). Antibodies used were as follows: β-catenin (SC-70509), GSK-3β (SC-9166), AKT (SC-5298), and β-actin (SC-47778) from Santa Cruz Biotechnology (Santa Cruz, CA); phospho-GSK-3β (ser 9) (#9323), phospho-AKT (ser 473) (#9271), phospho-mTOR (ser2448) (#2971), mTOR (#2972), phospho-S6 (ser235/236) (#2211), and S6 (#2217) from Cell Signaling (Beverly, MA); and active-β-catenin (#05-665) from Millipore (Temecula, CA). Secondary antibodies and enhanced chemiluminescence substrate were from Pierce (Rockford, IL).

Electron spin resonance (ESR) assay

All ESR measurements were conducted using a Bruker EMX spectrometer (Bruker Instruments, Billerica, MA) and a flat cell assembly, as described previously (Son et al., 2010a). A spin trap, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) was charcoal purified and distilled to remove all ESR detectable impurities before use. PBS was also purified with Chelex 100 to protect from transition metal ion contamination. The Acquisit program was used for data acquisitions and analyses (Bruker Instruments). The Cr(VI)-exposed cells for 3 months were harvested and mixed with DMPO (50 mM). The samples were then transferred to a flat cell for ESR measurement. Experiments were performed at room temperature and under ambient air.

Tumorigenicity assay

Athymic nude mice (NU/NU, 6-8 weeks old; Charles River) were housed in a pathogen-free room in the animal facilities at the Chandler Medical Center, University of Kentucky. All animals were handled according to the Institutional Animal Care and Use (IACUC) guidelines. Cr(VI)-stimulated cells (1×10^6 cells) were resuspended in serum-free medium with Matrigel basement membrane matrix (BD Biosciences) at a 1:1 ratio (total volume = 100 μ l) and subcutaneously injected into the flanks of nude mice (two sites/mouse). Body weight and tumor mass were measured every week for 4 months. Tumor volumes were determined using a caliper and calculated according to the following formula: $(\text{width}^2 \times \text{length})/2$. Finally, tumor tissues were dissected, weighed, and fixed with formalin for immunohistochemical staining. The implantation site containing a BD Matrigel plug, subcutaneous tissue, peritoneum, and skin was isolated to use as control tissue corresponding to the tumor tissue.

Immunohistochemical staining

Tumor tissues were fixed with 4% paraformaldehyde at room temperature for 24 h, embedded in paraffin, and sectioned (10 μ m thickness). The slides were deparaffinized and processed for immunohistochemical staining according to the VECTASTAIN ABC Kit protocol (Vector Laboratories, Burlingame, CA). Briefly, the sections were incubated with 3% H_2O_2 in distilled water to block endogenous peroxidase activity. After antigen retrieval, the sections were blocked with normal serum for 20 min and then incubated with primary antibodies for 1 h. A negative control was provided by incubating sections with nonspecific mouse or rabbit serum IgG at the same dilution as for the primary antibodies. After washing with PBS, the sections were incubated with biotinylated secondary antibodies for 30 min. The sections were then washed twice with PBS, incubated with ABC reagent for 30 min, and developed in DAB solution until the desired staining intensity was achieved.

Statistical analysis

All data are expressed as mean $\pm$ standard error (SE). One-way analysis of variance (ANOVA) using SPSS ver. 10.0 software was used for multiple comparisons. A p value of < 0.05 was considered statistically significant.

Results

Chronic exposure to Cr(VI) induces carcinogenic properties in BEAS-2B cells

The cell transformation assay is used as a predictive tool for carcinogenicity (Barrett et al., 1984). An anchorage-independent colony formation assay was performed 3 months after stimulation with Cr(VI). Continuous exposure of BEAS-2B cells to Cr(VI) induced a dose-dependent transformation of these cells, as shown by the marked increases in size and number of colonies compared with the vehicle control (Fig. 1A). Continuous exposure to 50 nM and 100 nM Cr(VI) increased the number of colonies to approximately 200/ 10^4 cells ($P < 0.05$) and 350/ 10^4 cells ($P < 0.01$), respectively, whereas few colonies were observed in the untreated control. Cr(VI)-stimulated clonogenicity was also demonstrated by a clonal assay, in which Cr(VI) treatment significantly increased the number of colonies in a dose-dependent manner (Fig. 1B). Furthermore, multiple foci formed on culture plates of Cr(VI)-exposed BEAS-2B cells (Fig. 1C). However, the passage-matched control cells did not exhibit these changes.

Chronic Cr(VI) stimulation increases cell migration and invasion of BEAS-2B cells

Continuous Cr(VI) stimulation induced the migration of BEAS-2B cells in a dose-dependent manner, such that treatment with 50 nM and 100 nM Cr(VI) increased cell migration up to

5.5 and 7.3-fold, respectively, compared with the vehicle control. The result of the Matrigel invasion assay also showed that chronic Cr(VI) stimulation increased the invasiveness of BEAS-2B cells in a dose-dependent manner (Fig. 2B). Treatment with Cr(VI) at 10, 50, and 100 nM increased cell invasiveness approximately 11-, 38-, and 52-fold, respectively, compared with the vehicle control.

Overexpression of antioxidant enzymes attenuates Cr(VI)-induced carcinogenic potential in BEAS-2B cells

To investigate whether intracellular ROS are involved in Cr(VI)-induced carcinogenesis, we generated BEAS-2B cells that overexpress CAT, SOD1, or SOD2 (Son et al., 2012). The results of the soft agar assay showed that Cr(VI)-stimulated formation of colonies was attenuated significantly by transfection with each of the antioxidant-specific vectors (Fig. 3A). This was partially consistent with results from the cell migration assay, in which transfection with SOD1 and SOD2, but not CAT, significantly diminished Cr(VI)-stimulated increase in cell migration (Fig. 3B). Similarly, transfection with CAT, SOD1, or SOD2 attenuated Cr(VI)-mediated cell invasion to approximately 55, 87, and 75%, respectively, compared with the vector controls (Fig. 3C).

Continuous exposure to low concentrations of Cr(VI) increases ROS levels and alters the cellular levels of antioxidant enzymes and anti-apoptotic proteins in BEAS-2B cells

The cells were incubated for 3 months with and without 100 nM Cr(VI), intracellular ROS levels were measured by recording electron spin resonance (ESR) spectra (Fig. 4A). These cultured cells without Cr(VI) exposure showed only trace amounts of ESR signal, whereas the cells exposed to Cr(VI) for 3 months clearly showed a 1:2:2:1 quartet ESR signal. However, we did not detect any significant increase in ROS levels when the cells were exposed to Cr(VI) at the same concentration for less than 3 months (data not shown). While the expression levels of antioxidant enzymes, such as catalase, SOD1, and SOD2, were downregulated in Cr(VI)-exposed cells for 3 months, the levels of Bcl-2 and Bcl-xL were proportionally upregulated by Cr(VI) in a dose-dependent manner in these exposed cells (Fig. 4B). Furthermore, the Cr(VI)-mediated increases in these anti-apoptotic proteins were inhibited either by overexpression of antioxidant enzymes (Fig. 4C) or by treatment of the cells with LY294002 (Fig. 4D). These results suggest that ROS play an important role in Cr(VI)-exposed cells and that the induction of the anti-apoptotic Bcl-2 family is ROS-mediated and PI3K/Akt-dependent.

Cr(VI) activates ROS-dependent AKT/GSK-3 β / β -catenin-mediated signaling and inhibits autophagy through mTOR signaling in BEAS-2B cells

To elucidate the molecular mechanisms involved in Cr(VI)-induced carcinogenesis, we measured the protein levels of several protein kinases using Western blot analysis. The levels of phosphorylated PI3K and its downstream target AKT increased after stimulation with Cr(VI) in a dose-dependent manner (Figs. 5A and E). The expression level of p-GSK-3 β (Ser 9) also increased markedly in Cr(VI)-exposed cells, and the highest level of p-GSK-3 β (Ser 9) was observed after treatment with 100 nM Cr(VI) (Figs. 5A and E). The cellular levels of active and total β -catenin proteins also increased in Cr(VI)-treated cells (Figs. 5A and E). In addition, levels of total and phosphorylated mTOR and its downstream target p-S6 were dramatically increased (Fig. 5A). The levels of p-PI3K, p-AKT, p-GSK-3 β , and active and total β -catenin were reduced by transfection with either CAT, SOD1, or SOD2 (Figs. 5B and F). These results suggest that ROS are key mediators in PI3K/AKT/GSK-3 β / β -catenin signaling as well as PI3K/AKT/mTOR signaling. Co-incubation with an AKT inhibitor, LY294002, or a β -catenin inhibitor, FH535, attenuated the Cr(VI)-induced increase in the levels of active and total β -catenin (Figs. 5C and G). In contrast, the addition of the GSK-3 β inhibitor SB216763 facilitated the Cr(VI)-stimulated induction of both active

and total β -catenin. These findings were consistent with the results of the soft agar assay, in which inhibitors specific to AKT and β -catenin almost completely prevented the formation of colonies, whereas GSK-3 β inhibitor accelerated colony formation (Fig. 5D). In addition, Cr(VI) inhibited autophagy in nutrient-free conditions (Fig. 6). Increased levels of LC3-II induced by serum starvation, which is a hallmark of autophagy, were attenuated in Cr(VI)-treated BEAS-2B cells. This result indicates that the inhibition of autophagy by Cr(VI) contributes to Cr(VI)-induced carcinogenesis.

Cr(VI) increases tumor growth and expression of AKT/GSK-3 β / β -catenin in a mouse xenograft model

The carcinogenic potential of Cr(VI) *in vivo* was investigated by the subcutaneous injection of nude mice with control cells or Cr(VI)-stimulated cells. To avoid any effect of cell density on tumor formation, we injected each site with 1×10^6 cells. Over a one-month post-inoculation period, no tumor formation was observed in the vehicle control. Only one mouse showed visible tumor formation throughout the experimental period. However, all mice injected with Cr(VI)-stimulated cells showed obvious tumor formation, and the tumor size increased with time (Figs. 7A and B). Body weight gain was not different between the control and experimental groups during the experimental period. Western blot analysis revealed that levels of p-PI3K, p-AKT, p-GSK-3 β , and active β -catenin were much higher in the tumor tissues that formed at the injection sites of the xenograft mice in comparison to corresponding tissues from the control mice (Fig. 7D). Similarly, high expression levels of carcinogenesis marker proteins, such as cmyc, cyclooxygenase-2 (COX-2), HIF-1 α , MMPs, and VEGF were detected in tumor tissues compared with flank tissues of the control mice (Fig. 7E). The upregulation of anti-apoptotic proteins (Bcl-2 and Bcl-xL) were also shown in tumor tissues (Fig. 7E). Immunohistochemical staining confirmed that the levels of total and active β -catenin proteins were markedly higher in tumor tissues than in the controls (Fig. 7F). In addition, Cr(VI)-mediated tumor formation was attenuated by overexpression of catalase or SOD (Fig. 7G).

Discussion

Chromium (Cr(VI)) compounds are established carcinogens. Industrial exposure to these compounds is associated with a higher incidence of human lung cancer (Hayes, 1988; Langard, 1990). Environmental exposure to Cr(VI) could induce lung toxicity in the short term and carcinogenicity over the long term (Freeman et al., 1997). Both industrial and environmental exposure to Cr(VI) are associated with an increased incidence of cancers of the respiratory system (De Flora et al., 1990; Freeman et al., 1997; Hayes, 1988; Langard, 1990; Singh et al., 1998). Although the carcinogenic property of Cr(VI) is well recognized, the precise mechanisms underlying its carcinogenic activity are not fully understood.

Carcinogenesis is a multi-step process requiring long-term exposure to a carcinogen. The concentration of Cr(VI) is in the pico- to nanomolar concentration range in occupational and environmental exposure (OSHA, 2006). To mimic conditions similar to occupational and biologically relevant Cr(VI) exposure, we exposed human bronchial epithelial cells to Cr(VI) at nanomolar concentrations for 3 months. Cr(VI) concentrations from 0 to 100 nM are highly relevant to occupational and environmental exposure. Long-term exposure to low doses of Cr(VI) appeared to induce a pre-neoplastic state in cells, as the number of colonies formed in soft agar was significantly higher in Cr(VI)-stimulated cells than in unstimulated controls (Fig. 1A). This was consistent with the results of the clonal assay, in which chronic exposure of cells to Cr(VI) increased the number of colonies in a dose-dependent manner (Fig. 1B). Furthermore, cells stimulated with Cr(VI) over 3 months formed colonies on culture plates when the cells reached high confluency (Fig. 1C). Exposure of BEAS-2B cells to Cr(VI) at concentrations greater than 100 nM produced a cytotoxic effect such that viable

cells were rarely observed during the long culture period. Moreover, colony formation in the soft agar assay did not notably increase unless the cells were exposed to Cr(VI) for more than 2 months. Based on these observations, we used a maximum Cr(VI) concentration of 100 nM and an exposure period of up to 3 months.

Cell migration and invasion are hallmark characteristics of tumor metastasis. Chronic stimulation of BEAS-2B cells with Cr(VI) resulted in a marked increase in cell migration and invasion compared with unstimulated cells (Fig. 2). We also found that the expression levels of MMPs and VEGF in engrafted tumors were higher than in the tissues of control mice (Fig. 7E). Other markers of carcinogenesis, including c-myc, COX-2, and HIF-1 α , were also markedly expressed in the engraft tumors (Fig. 7E). These data suggest that Cr(VI) has the potential to induce metastasis. In contrast to the control mice, the inoculation of mice with Cr(VI)-stimulated cells induced the formation of tumors that gradually increased in volume (Figs. 7A and B). Additional experiments revealed that, compared with tumor xenografts generated by injection of transformed cells, these tumors were not aggressive (data not shown). Nonetheless, our present findings suggest that Cr(VI) not only induces malignant transformation *in vitro*, but also induces tumor formation *in vivo*.

ROS generation during the reduction process of Cr(VI) is considered to be responsible for the carcinogenicity of these compounds (Shi et al., 1994; Shi et al., 1998; Shi and Dalal, 1989; Shi and Dalal, 1990; Wang et al., 2000; Ye et al., 1995). However, the determination of the involvement of ROS signaling in metal carcinogenesis over the last decade was challenging, as ROS have dual functions in metal carcinogenesis. For instance, modest levels of ROS are required for cell transformation and carcinogenesis (Chang et al., 2010; Wang et al., 2011), whereas excessive levels induce apoptosis (Pan et al., 2010; Son et al., 2010a; Son et al., 2010b). Recently, our research group found that ROS signaling was involved in As-(Chang et al., 2010; Zhang et al., 2011), Cr-(Wang et al., 2011), and Cd-(Son et al., 2012) mediated carcinogenesis. However, how ROS-mediated signaling leads to the transformation of normal cells into cancer cells is not clear.

To verify the involvement of ROS in Cr(VI)-induced carcinogenesis, we observed ROS levels after Cr(VI) exposure to cells for 3 months. The generation of ROS was detected directly using ESR (Fig. 4A), and the results indicated that Cr(VI) generates ROS in cells even at nanomolar concentrations. Cr(VI)-induced colony formation, cell migration, and invasion were inhibited by overexpression of each of the antioxidant enzymes (Fig. 3). These results further support the involvement of ROS in Cr(VI)-induced carcinogenesis. In particular, transfection with SOD2 showed the most prominent suppression of Cr(VI)-induced colony formation and cell migration (Figs. 3A and B). However, Cr(VI)-induced cell invasion was inhibited to a greater extent in cells transfected with SOD1 than in other cells (Fig. 3C). Furthermore, inoculation of cells overexpressing CAT or SOD1 reduced the formation of tumors in mice (Fig. 7G), although the injection of cells overexpressing SOD2 had no effect. It is assumed that the effect of ROS in carcinogenesis differs *in vitro* and *in vivo* as well as in the cancer microenvironment. It is also likely that ROS are only involved in the initiation of metal-induced carcinogenesis, rather than in other stages of carcinogenesis, as ROS levels are lower in metal-transformed cells than in normal cells (Chang et al., 2010). Although it is commonly accepted that intracellular ROS levels are higher in cancer cells than in normal cells (Trachootham et al., 2009), some studies have reported the opposite phenomenon, where ROS levels were lower in cancer cells than in normal cells (Diehn et al., 2009; Jang and Sharkis, 2007). Actually, our preliminary study demonstrated that the basal ROS levels of metal-transformed cells such as Cr-, As-, Ni-, or Cd-transformed cells were lower than passage matched BEAS-2B cells in ESR experiment (data not shown). This led us to postulate that ROS are required for the initiation of carcinogenesis in Cr(VI)-exposed cells.

We next determined how ROS signaling is involved in the transformation of normal cells to cancer cells by examining the role of the β -catenin pathway. The β -catenin pathway is required for the development of leukemia stem cells (Wang et al., 2010), colon carcinomas (Behrens, 2005; Lustig and Behrens, 2003; Zhang et al., 2011), and other cancer types (Klaus and Birchmeier, 2008; Lustig and Behrens, 2003). It has also been reported that β -catenin regulates a diverse set of oncogenes, including *c-myc*, *cyclin D1*, *ABCB1* (Chakraborty et al., 2010), *VEGF* (Zhang et al., 2001), *COX-2* (Howe et al., 2001), and *MMPs* (Marchenko et al., 2002). β -catenin is tightly regulated by the Axin-APC-GSK-3 β complex; phosphorylation of β -catenin by GSK-3 β leads to its degradation through a ubiquitin-proteasome pathway (Lustig and Behrens, 2003) (Nakamura et al., 1998). Our present findings revealed that levels of both total and active β -catenin (dephosphorylated β -catenin) were markedly increased in Cr(VI)-stimulated cells, as well as in the tumor tissues of mice injected with Cr(VI)-stimulated cells (Figs. 5A and 7D). These results suggest that β -catenin regulates the expression of target genes involved in cell transformation and carcinogenesis. Further, β -catenin stabilization seems to be regulated by GSK-3 β in Cr(VI)-induced carcinogenesis, based on the increase in p-GSK-3 β levels in Cr(VI)-stimulated cells and in xenograft mice injected with these cells (Figs. 5A and 7D). This is consistent with the increased phosphorylation of PI3K and AKT in Cr(VI)-stimulated cells. It is likely that phosphorylation of GSK-3 β at Ser 9 by AKT inhibits GSK-3 β activity and leads to β -catenin stabilization during Cr(VI)-induced carcinogenesis.

To clarify the roles of ROS in signal transduction pathways involved in Cr(VI)-induced carcinogenesis, cells expressing antioxidant enzymes were stimulated with 100 nM Cr(VI). Transfection with CAT, SOD1, or SOD2 suppressed the phosphorylation levels of signaling molecules, and also reduced levels of total and active β -catenin (Figs. 5B and F). Similarly, pharmacological inhibitors of AKT and β -catenin not only attenuated the induction of total and active β -catenin, but also prevented an increase in the number of colonies after Cr(VI) exposure (Figs. 5C and D). In contrast, the blockage of GSK-3 β activation by SB216763 enhanced colony formation in Cr(VI)-exposed cells (Fig. 5D). These results strongly suggest that ROS activate PI3K/AKT/GSK-3 β / β -catenin signaling in Cr(VI)-induced carcinogenesis; thus, regulation of this signaling may allow for the control of carcinogenic processes. Our results also indicate that Cr(VI)-mediated activation of β -catenin signaling leads to increased expression of *c-myc*, *COX-2*, *HIF-1 α* , *MMPs*, and *VEGF* during carcinogenesis (Fig. 7E). The inhibition of apoptosis and autophagy are important processes during carcinogenesis (Jin and White, 2007; Moscat and Diaz-Meco, 2009), as cancer cells inhibit apoptosis and prolong their survival (Vivanco and Sawyers, 2002). Autophagy has a tumor suppression function in human breast, ovarian, and prostate cancer (Liang et al., 1999). Chronic exposure to Cr(VI) at nanomolar concentration appears to inhibit apoptosis. Our data showed that expression of antiapoptotic proteins such as Bcl-2 and Bcl-xL increased during Cr(VI) exposure (Fig. 4B), and both the Cr(VI)-exposed and Cr(VI)-transformed BEAS-2B cells demonstrated resistance against apoptosis in comparison to control cells (data not shown). Furthermore, the expression of anti-apoptotic proteins increased in Cr(VI)-stimulated cells injected into mouse xenograft tumors (Fig. 7E). The activation of antiapoptotic proteins may be due to ROS-dependent activation of AKT in Cr(VI)-exposed cells (Figs. 4C and D). It has reported that the phosphorylation of Akt by stimuli activates the induction of antiapoptotic proteins in numerous cell types (Kumar et al., 2009; Preuss et al., 2010; Raina et al., 2004). Chronic exposure to Cr(VI) also inhibited autophagy under serum deprivation conditions (Fig. 6). The inhibition of autophagy by Cr(VI) occurred mainly through the mTOR pathway. These results suggest that apoptosis and autophagy are inhibited via the promotion of cellular survival mechanisms induced by Cr(VI) exposure, and this eventually contributes to Cr(VI)-induced carcinogenesis. It should be note that BEAS-2B is an SV40 T antigen immortalized bronchial epithelial cell line (Reddel et al., 1988). It has reported that SV40 large-T antigen was able to bind and

inactivate both p53 and retinoblastoma (RB) proteins (Bargonetti et al., 1992). The inactivation of p53 by SV40 T antigen may enhance anti-apoptotic properties of Cr(VI)-exposed BEAS-2B cells and promote carcinogenesis. Further studies will be needed to investigate the p53 function in metal-induced cell transformation of BEAS-2B cells. Taken together, the current findings demonstrate that ROS-dependent activation of PI3K/AKT/GSK-3 β / β -catenin signaling is critical for Cr(VI)-induced carcinogenesis, as well as for the inhibition of apoptosis and autophagy (Fig. 8).

In summary, the present findings demonstrate that ROS play an important role in the activation of signaling cascades involving PI3K/AKT/GSK-3 β / β -catenin in the Cr(VI)-mediated transformation of epithelial cells. Increases in the levels of total and active β -catenin stimulate the expression of target genes, such as *c-myc*, *COX-2*, *HIF-1 α* , *MMPs*, and *VEGF*, which might be critical events in Cr(VI)-induced carcinogenesis. The inhibition of apoptosis and autophagy through the PI3K/AKT-mediated overexpression of Bcl-2/Bcl-xL and mTOR, respectively, contributes to Cr(VI)-induced carcinogenesis. Collectively, our results implicate ROS and ROS-dependent signaling molecules as useful therapeutic targets for Cr(VI)-induced human lung cancer.

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Abbreviations

ROS	reactive oxygen species
CAT	catalase
SOD	superoxide dismutase
MMPs	matrix metalloproteinases
VEGF	vascular endothelial growth factor
COX-2	cyclooxygenase-2
HIF-1α	hypoxia-inducible transcription factor-1 α
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
LC3	microtubule-associated protein 1 light chain 3
mTOR	mammalian target of rapamycin
DMPO	5,5-dimethyl-1-pyrroline-1-oxide
ESR	electron spin resonance

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Highlights

- Chronic exposure to Cr(VI) induces carcinogenic properties in BEAS-2B cells.
- ROS play an important role in Cr(VI)-induced tumorigenicity of BEAS-2B cells.
- PI3K/AKT/GSK-3 β / β -catenin signaling involved in Cr(VI) carcinogenesis.
- The inhibition of apoptosis and autophagy contributes to Cr(VI) carcinogenesis.

Fig. 1.

Cr(VI) induces colony formation in BEAS-2B cells. After exposure of cells to increasing concentrations (0-100 nM) of Cr(VI) for 3 months, soft agar (A) and clonal assays (B) were performed. Continuous exposure to low concentrations of Cr(VI) also induces formation of multiple foci in cultured BEAS-2B cells (C). Representative images from three independent experiments are shown. The number of colonies is given in the lower panel. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. vehicle control.

Fig. 2.

Cr(VI) increases potential of cells to migrate and invade. A cell migration and invasion assay was performed using transwell chambers. (A) The top chambers of the transwells were loaded with Cr(VI)-stimulated cells and incubated for 12 h. (B) Cr(VI)-stimulated cells were loaded into the pre-coated Matrigel upper chambers and incubated for 3 days. Migrated or invaded cells were fixed, stained with hematoxylin solution, and counted under a microscope. Results are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. vehicle control.

Fig. 3.

Overexpression of antioxidant enzymes attenuates Cr(VI)-induced carcinogenicity of BEAS-2B cells. CAT-Myc-DDK-, SOD1-Myc-DDK-, and SOD2-EGFP-tagged plasmids were transfected into BEAS-2B cells. Cells overexpressing each of the antioxidant enzymes were stimulated with Cr(VI) for 3 months and soft agar (A), cell migration (B), and cell invasion assays (C) were performed. Results are representative of three independent experiments. ** $P < 0.01$ and *** $P < 0.001$ vs. vehicle control. # $P < 0.05$ and ## $P < 0.01$ vs. Cr(VI)-exposed vector control.

Fig. 4.

Continuous exposure to a low concentration of Cr(VI) increases ROS levels, and upregulation of anti-apoptotic proteins levels is ROS-mediated and PI3K/AKT-dependent in BEAS-2B cells. (A) Electron spin resonance (ESR) spectra were recorded for cells exposed to Cr(VI) (100 nM) for 3 months. The generation of a 1:2:2:1 quartet ESR signal is shown (upper panel) and the signal intensity of DMPO-OH is represented (lower panel). (B) The expression of antioxidant enzymes and anti-apoptotic proteins was analyzed by Western blotting. The expression of Bcl-2 and Bcl-xL was shown in Cr(VI)-exposed antioxidant-overexpressing cells (C) and in cells co-incubated with LY294002 cells (D). * $P < 0.05$ vs. vehicle control. The ESR spectrometer settings were as follows: frequency, 9.8 GHz; power, 39.91 mW; modulation frequency, 100 kHz; receiver gain, 5.02×10^5 ; time constant, 40.96 ms; modulation amplitude, 1.00 G, scan time, 60 s; and magnetic field, 3451 ± 100 G. All spectra shown are an accumulation of 16 scans.

Fig. 5.

Cr(VI) leads to ROS-dependent activation of PI3K, AKT, mTOR, GSK-3 β , and β -catenin signaling in BEAS-2B cells. Using Western blots, we analyzed the protein levels of these factors in cells stimulated with increasing concentrations of Cr(VI) (0-100 nM) (A), or in cells transfected with CAT, SOD1, or SOD2 treated with 100 nM Cr(VI) for 3 months (B). BEAS-2B cells were also exposed to 100 nM Cr(VI) in the presence of LY294002 (2 μ M), SB216763 (1 μ M), or FH535 (2 μ M) for 3 months, and protein levels and colony formation ability were analyzed by Western blotting (C) and soft agar assay (D), respectively. Colony formation was photographed at $\times 40$ magnification (D, upper panel) and the number of colonies is shown (D, lower panel). The band intensity of each Western results was expressed as the mean $\pm$ SE relative to the control of triplicate experiments and represented in (E), (F), and (G). * P < 0.05, ** P < 0.01, and *** P < 0.001 vs. vehicle control. # P < 0.05 and ## P < 0.01 vs. Cr(VI)-exposed control.

Fig. 6.

Cr(VI) inhibits autophagy in nutrient-free conditions. Cr(VI)-exposed BEAS-2B cells (0.1×10^6 cells/well) were seeded in a 6-well plate. After overnight incubation, the cells were starved for 4 days in serum-free media. Afterwards, the cells were treated with LY294002 (10 μM), SB216763 (10 μM), FH535 (20 μM), or rapamycin (100 nM) in the presence of 100 nM Cr(VI) for 12 h. The expression levels of proteins were analyzed by Western blotting and the band intensity of LC3-II was represented. ** $P < 0.01$ vs. 10% FBS control. # $P < 0.05$ and ## $P < 0.01$ vs. serum free control.

Fig. 7.

Cr(VI) increases tumor growth and expression of carcinogenic markers in a xenograft animal model. Cr(VI)-exposed BEAS-2B cells (1×10^6 cells/site) were injected subcutaneously into 6-week-old male athymic nude mice. (A) – (C) indicate xenograft tumor, tumor volumes, and body weight gained, respectively. In addition, tumor tissues were dissected and processed for Western blot analysis (D and E) or immunohistochemical staining (F). The control and antioxidant-overexpressing cells were exposed to Cr(VI) and adjusted to perform the xenograft assay (G). * $P < 0.05$ vs. vehicle control. # $P < 0.05$ vs. Cr(VI)-exposed control.

Fig. 8.

Proposed model of Cr(VI)-induced carcinogenesis. Chronic exposure of BEAS-2B cells to Cr(VI) increases ROS levels, which activates PI3K/AKT signaling, leading to phosphorylation of GSK-3β. Phosphorylated GSK-3β dephosphorylates β-catenin and induces its translocation into the nucleus, which results in the expression of target genes such as *c-myc*, *COX-2*, *HIF-1α*, *MMPs*, and *VEGF*. The inhibition of apoptosis or autophagy through the PI3K/AKT-mediated Bcl-2/Bcl-xL or mTOR pathway also enhances carcinogenesis in Cr(VI)-exposed BEAS-2B cells.