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The Dual Roles of c-Jun NH_2-Terminal Kinase Signaling in Cr(VI)-Induced Apoptosis in JB6 Cells

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Occupational exposure to chromium (Cr) compounds has been shown to cause serious toxic and carcinogenic effects. The skin is an important target for the compounds in industrially exposed Cr workers. c-Jun NH_2-terminal kinase (JNK) regulates cell proliferation, apoptosis, and differentiation. This protein's effects on cellular response depend upon the cell type and stimuli. The mechanisms by which hexavalent chromium (Cr(VI)) leads to apoptosis in the skin are unclear at present. The aim of this study is to examine whether JNK regulates apoptosis in Cr(VI)-exposed mouse JB6 epidermal cells. The present study showed that Cr(VI) induced apoptotic cell death through JNK activation. The blockage of JNK by small interference RNA (si-RNA) transfection suppressed Cr(VI)-induced apoptotic cell death with the concomitant downregulation of antiapoptotic Bcl-2 family proteins, mitochondrial membrane depolarization ($\Delta\psi m$), caspase activation, and poly (ADP-ribose) polymerase cleavage. However, inhibition of c-Jun expression by si-RNA transfection enhanced cytotoxicity, which corresponded to increasing apoptosis and $\Delta\psi m$. This phenomenon is associated with p53 activation caused by increasing reactive oxygen species (ROS) levels because of the downregulation of superoxide dismutase expression in si-c-Jun–transfected cells. Taken together, Cr(VI) induces apoptosis via JNK-mediated signaling, whereas c-Jun activation acts as an inhibitor of apoptotic signaling. Additionally, ROS generated by Cr(VI) is a pivotal regulator of JNK.

Key Words: **apoptosis; Cr(VI); JNK; c-Jun; ROS.**

Chromium (Cr) compounds are widely used in industry and have serious toxic and carcinogenic effects on animals and humans (Cohen *et al.*, 1993; Costa, 1997). Hexavalent chromium (Cr(VI)) enters the body by inhalation, ingestion, or absorption through the skin. For occupational exposure, the airways and skin are the primary routes of uptake (Costa, 1997; Shelnutt *et al.*, 2007). Upon entering the cell, Cr(VI) is reduced by cellular reductants to its lower oxidation states, pentavalent chromium and tetravalent Cr (Shi *et al.*, 1999). These intermediate states of Cr are reactive and can produce reactive oxygen species (ROS) (Shi and Dalal, 1989, 1990c), which cause DNA strand breaks, base modification, lipid peroxidation, and transcription factor activation (Ding and Shi, 2002; Hodges *et al.*, 2001; Shi *et al.*, 1999; Stohs *et al.*, 2000; Xu *et al.*, 1992; Ye *et al.*, 1995). Occupational exposure to Cr(VI) is a well-established cause of skin damage such as skin ulcers, irritant contact dermatitis, and delayed hypersensitivity reactions (Hansen *et al.*, 2006; OSHA, 2006; Shelnutt *et al.*, 2007). The mildest skin reactions consist of erythema, edema, papules, and vesicles (OSHA, 2006). Cr(VI) also causes allergenic skin eczema, atopic dermatitis (Hansen *et al.*, 2006), and dermal toxicity (Joseph *et al.*, 2008; Shelnutt *et al.*, 2007).

Cellular events such as cell proliferation, apoptosis, and differentiation are tightly regulated by mitogen-activated protein kinases (MAPK) including c-Jun NH_2-terminal kinase (JNK), extracellular signal regulated kinase (ERK), and p38 kinase (Liu *et al.*, 2002; Tobiume *et al.*, 2001). ROS are critical mediators of cellular signaling, which regulate many cellular processes where MAPK signaling is closely involved (Clerk *et al.*, 1998; Liu *et al.*, 2002; Son *et al.*, 2009, 2010b; Wang *et al.*, 1998). In particular, JNK-mediated signaling is important in modulating apoptosis signaling in response to oxidative stress (Chang and Karin, 2001; Wang *et al.*, 1998). JNK acts as an upstream effecter of transcription factor activator protein-1 (AP-1) (Derijard *et al.*, 1994; Kyriakis *et al.*, 1994; Ventura *et al.*, 2003). AP-1 is a multimeric protein consisting of Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra1, and Fra2) subunits. AP-1 binds to the TRE (TPA DNA response element) and regulates many kinds of early response gene expression (Munoz *et al.*, 1996). AP-1 also mediates either cell survival or death, which are caused through varying combinations of Jun subproteins (Leppa and Bohmann, 1999). Importantly, c-Jun is the major phosphorylation target of JNK (Davis, 1995).

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We recently showed that Cr(VI) induced mitochondrial-mediated and caspase-dependent apoptosis in epidermal cells through activation of p53, which was mainly mediated by ROS generated by the chemical (Son *et al.*, 2010a). This supports the suggestion that ROS could play a crucial role in Cr(VI)-mediated cell death. There is also considerable evidence emphasizing the involvement of MAPK-mediated signaling in Cr(VI)-induced apoptosis. Actually, Cr(VI) activates JNK, ERK, and/or p38 in rat H4 hepatoma, murine embryonic stem cells, and human bronchial epithelial or lung carcinoma cell line (CL) 3 cells (Chen *et al.*, 2009; Chuang *et al.*, 2000; Kim and Yurkow, 1996; Samet *et al.*, 1998). These findings and our previous data indicate that among MAPK, JNK has important roles in Cr(VI)-treated cells because JNK is activated sensitively in the presence of ROS (Ge *et al.*, 2008; Shen and Liu, 2006; Son *et al.*, 2009, 2010b). It is of note that almost all the previous studies have used high concentrations of Cr(VI) over short exposure times, as opposed to the chronic exposure to low doses of Cr(VI) which occurs occupationally. Moreover, the cellular mechanisms by which JNK mediates apoptosis in Cr(VI)-treated cells remain unclear. The present study has further examined the possible role of JNK in regulating cellular responses to low concentrations of Cr(VI) in skin epidermal JB6 cells. Here, we demonstrate the critical roles of JNK-mediated signaling in facilitating ROS-mediated apoptosis in Cr(VI)-exposed JB6 cells.

MATERIALS AND METHODS

Chemicals and laboratory wares. Unless specified otherwise, all chemicals and laboratory wares were purchased from Sigma Chemical Co. (St Louis, MO) and Falcon Labware (Bectone-Dickinson, Franklin Lakes, NJ). Eagle's minimal essential medium (EMEM), fetal bovine serum (FBS), gentamicin, and L-glutamine were purchased from Gibco Co. (Gibco BRL, NY). Dihydroethidium was obtained from Molecular Probes (Eugene, OR).

Cell culture and treatment. The JB6 mouse epidermal cell line, Cl 41 (American Type Culture Collection, CRL-2010), and cells stably transfected with the AP-1-luciferase reporter plasmid (JB6/AP-1 cells) were cultured in EMEM containing 5% FBS, 2mM L-glutamine, and 50 μg of gentamicin per milliliter. The cells were grown at 37°C in a 5% CO_2 atmosphere. The monolayer cells were trypsinized, and 1 million cells per milliliter were resuspended in either 3 ml or 200 μl of the media and spread onto either 60-mm culture dish or 96-well plates, respectively. After the cells had reached 80% confluence, the culture medium was replaced with fresh medium containing 0.5% FBS and various concentrations of $Na_2Cr_2O_7$ (0–10μM).

Plasmids and transfection. The SOD1-Myc-DDK– and SOD2-enhanced green fluorescent protein–tagged plasmids were purchased from Origene (Rockville, MD) and Addgene (Cambridge, MA), respectively. Transfection was performed using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacture's protocol. Briefly, JB6 cells were seeded in six-well culture plates and transfected with 4 μg of plasmids at approximately 50% confluency. Overexpressed cellular levels of the proteins specific for Cu/Zn superoxide dismutase (SOD) (SOD1) or MnSOD (SOD2) were checked by immunoblotting, and the cells were maintained using G418 for stable cell lines.

Measurement of cell viability. 3-(4,5-Dimethylthiazol-2yl-)-2,5-diphenyl tetrazolium bromide (MTT) was used to evaluate viability of cells. After transfection with small interference RNA (si-RNA), JB6 cells were treated with or without 10μM of $Na_2Cr_2O_7$ for 48 h. Thereafter, 5 μl of an MTT solution (5 mg/ml in PBS as stock solution) was added into each well of 96-well plates and incubated for 4 h at 37°C. MTT-reducing activity of cells was measured by treating them with acidic isopropanol prior to reading at 560 nm with Spectra Max Plus^{384} (Molecular Devices, Sunnyvale, CA).

FITC-*Annexin V/propidium iodide staining.* JB6 cells were washed twice with PBS before being suspended in 1× binding buffer (10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 140mM NaOH, and 2.5mM $CaCl_2$). Five microliters of fluorescein isothiocyanate (FITC)-labeled Annexin V were mixed with 100 μl of cell suspension containing 1×10^5 cells, and the cells were incubated at room temperature for 10 min. Afterward, 2 μl of propidium iodide (PI) solution (10 μg/ml) was added into the cells followed by additional 10 min incubation on ice. The scatter parameters of the cells (20,000 cells per experiment) were analyzed using a Fluorescence-Activated Cell Sorter Calibur system. Four cell populations were identified according to the following interpretation: viable population in the lower left quadrant (low PI and FITC signals), early apoptotic population in the lower right quadrant (low PI and high FITC signals), necrotic population in the upper left quadrant (high PI and low FITC signals), and late apoptotic or necrotic population in the upper right quadrant (high PI and high FITC signals).

Measurement of mitochondrial membrane potential. The mitochondrial membrane potential ($\Delta\Psi m$) was monitored using 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), a lipophilic cationic fluorescence dye. JC-1 is capable of selectively entering mitochondria, where it forms monomers, and emits green fluorescence (FL-1) when $\Delta\Psi m$ is relatively low. At a high $\Delta\Psi m$, JC-1 aggregates and gives red fluorescence (FL-2) (Cossarizza *et al.*, 1993). Thus, the red and green fluorescence of JC-1 reflect the change of $\Delta\Psi m$ of the mitochondrial membrane. Briefly, cells (0.5×10^6 cells per well) were seeded into 60-mm culture dishes and transfected with si-RNA and the JB6 cells were cultured in MEM supplemented with or without 10μM of $Na_2Cr_2O_7$ for 48 h. Cells were trypsinized, washed in ice-cold PBS, and incubated with 2μM JC-1 at 37°C for 20 min. Finally, cells were washed twice with PBS and analyzed by flow cytometry.

Western blot analyses. Cell lysates were made in lysis buffer (50mM Tris-Cl, pH 7.4, 1mM EDTA, 150mM NaCl, 1% Nonidet P-40, 0.25% Na-deoxycholate, and 1 μg/ml of aprotinin, leupeptin, and pepstatin). Equal amounts of protein (20 micrograms per sample) were separated by the NuPAGE Bis-Tris electrophoresis system (Invitrogen) and blotted onto nitrocellulose membrane (Whatman, Dassel, Germany). Blots were probed with primary antibodies for either 2 h at room temperature or overnight at 4°C before incubation with horseradish peroxidase–conjugated anti-IgG in a blocking buffer for 1 h. Polyclonal antibody specific for SOD1 (SC-11407) and monoclonal antibodies specific for β-actin (SC-47778) and p-c-Jun NH_2-terminal kinase (p-JNK) (SC-6252) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bcl-2 (2876), Bcl-xL (2762), Bid (2003), p-p53 (Ser 15) (9284), caspase-8 (4927), c-Jun (9162), p-c-Jun (9261), JNK (9252), cleaved caspase-3 (Asp 175) (9661), and glutathion peroxidase1 (GPX1) (3206) polyclonal antibodies were purchased from Cell Signaling (Beverly, MA). The monoclonal antibodies specific to p53 (OP03) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (A00839-40) and polyclonal antibody specific to Cu/ZnSOD (07403) were purchased from Oncogene, Genscript, and Millipore, respectively. The poly (ADP-ribose) polymerase (PARP) (SA-249) and catalase (CAT) (NB100–79910) antibodies were purchased from Biomol (Plymouth Meeting, PA) and Novus (Littleton, CO), respectively. Secondary antibodies and enhanced chemiluminescence substrate were from Pierce (Rockford, IL). The blots were exposed to Hyperfilm (Amersham Pharmacia Biotech), and bands were quantified with ImageJ densitometry software (National Institutes of Health, Bethesda, MD).

Measurement of cellular superoxide anion ($O_2^{\cdot-}$) production. Dihydroethidium was used for staining $O_2^{\cdot-}$, which are produced by intact cells (Qian *et al.*, 2003; Zamzami *et al.*, 1995). Briefly, cells (0.2×10^6 cells per well)

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were seeded into six-well culture plates and transfected with si-RNA and the JB6 cells were cultured in the presence or absence of 10μM of $Na_2Cr_2O_7$ for 24 h. Finally, the cells were exposed to dihydroethidium at a final concentration of 2μM for 30 min and processed for flow cytometric analysis.

Electron spin resonance assay. All electron spin resonance (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, was charcoal purified and distilled to remove all ESR detectable impurities before use. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate and 1,1-diphenyl-2-picrylhydrazyl as reference standards. The Acquisit program was used for data acquisitions and analyses (Bruker Instruments). Reactants were mixed in test tubes to a total final volume of 0.5 ml. The reaction mixture was then transferred to a flat cell for ESR measurement. Experiments were performed at room temperature and under ambient air.

si-RNA transfection. Silencer predesigned si-RNA for mouse *JNK1* (si-RNA ID: 188653), *JNK2* (si-RNA ID: 73119), *p53* (si-RNA ID: 187425), *c-Jun* (si-RNA ID: 155944), negative control (AM4611), and positive control *GAPDH* (si-RNA ID: 4390849) were obtained from Ambion (Austin, TX). JB6 cells were seeded in 96- or six-well culture plates and transfected at approximately 50% confluency with the si-RNA duplexes using Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer's instructions. Medium was changed after 6 h to minimize cytotoxicity. Cellular levels of the proteins specific for the si-RNA transfection were checked by immunoblotting, and all experiments were performed 24 h after transfection.

Assay of AP-1 activity. Stably transfected JB6 cells with AP-1-luciferase reporter plasmid were kindly provided from Dr Min Ding (National Institute for Occupational Safety and Health, Morgantown, WV). When JB6/AP-1 cells reached 80–90% confluence, the cells were incubated in a low serum (0.5% FBS) containing EMEM for 24 h to minimize basal AP-1 activity. The cells were exposed to various concentrations of $Na_2Cr_2O_7$ (0–10μM) for 24 h. The cells were also exposed to $Na_2Cr_2O_7$ (10μM) for various times (0 h–24 h). The luciferase activity specific to AP-1 was then measured by using a luciferase assay kit (Promega, Madison, WI) as described elsewhere (Ding *et al.*, 1999). The results were expressed as relative AP-1 activity compared with control.

Statistical analysis. All the data have been expressed as mean ± SE. One-way ANOVA using SPSS version 10.0 software was used for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Cr(VI) Leads to Activation of JNK in Skin Epidermal Cells

A 24-h treatment of cells with Cr(VI) resulted in increased levels of phosphorylated JNK in a dose-dependent manner (Fig. 1A). This JNK activation was decreased after 48 h incubation when the cells treated with 10μM of $Na_2Cr_2O_7$ (Fig. 1B). For further verification of JNK's involvement, we transfected the cells with si-RNAs specific to JNK1 or JNK2, by which the expression of both proteins was suppressed (Fig. 1C). The Cr(VI)-induced decrease in cell viability was attenuated significantly in the cells transfected with either si-JNK1 ($p < 0.05$) or si-JNK2 ($p < 0.01$) (Fig. 1D). Treatment with 10μM $Na_2Cr_2O_7$ for 48 h in cells transfected with si-JNK1 and si-JNK2 increased the MTT-reducing activity to 43 and 51%, respectively, compared with the 10μM $Na_2Cr_2O_7$-treated cells (33%). These results indicated that JNK-mediated signaling is involved in Cr(VI)-induced cell death in JB6 cells.

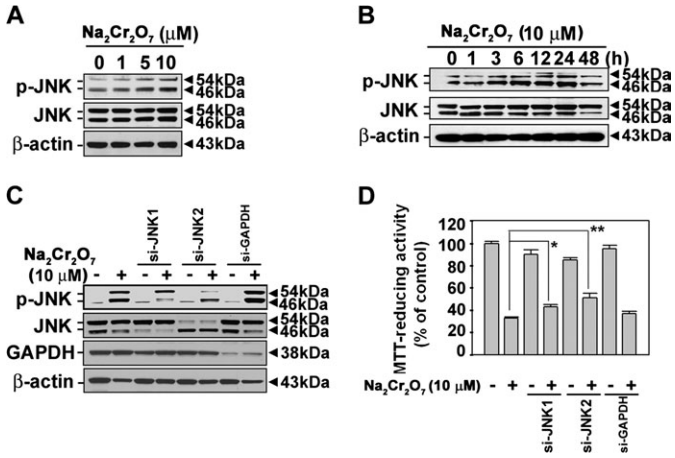

FIG. 1. JNK signaling is involved in Cr(VI)-induced cell death in JB6 cells. (A) JB6 cells were incubated with $Na_2Cr_2O_7$ at various concentrations (0–10μM) for 24 h. (B) Cells were exposed to 10μM $Na_2Cr_2O_7$ for various times (0–48 h). Protein lysates were analyzed by a NuPAGE Bis-Tris electrophoresis system, and the levels of phosphorylated JNK and total JNK were detected by Western blot analysis. JB6 cells were transfected with si-RNAs specific to JNK1 or JNK2. After 24 h incubation, they were exposed to 10μM $Na_2Cr_2O_7$. Finally, the protein levels were analyzed by Western blot at 24 h (C), and MTT assay was performed at 48 h (D). β-Actin and GAPDH were used as control protein and control transfection, respectively. The results are shown as the mean ± SE of three separate experiments. $*p < 0.05$ and $**p < 0.01$ versus the $Na_2Cr_2O_7$ treatment alone (ANOVA, Scheffe's test).

JNK Activation Is Closely Involved in Cr(VI)-Induced Apoptosis and Mitochondria Stress

In order to clarify the involvement of JNK in Cr(VI)-induced cytotoxicity, various apoptosis detection assays were subsequently performed. Cr(VI) treatment resulted in the induction of cleaved caspase-3 and the decrease of cellular Bcl-2 and Bcl-xL levels, as expected. In addition, decrease of procaspase-8 and increase of cleavage of Bid and PARP proteins were observed in the cells. However, these changes in Cr(VI)-exposed cells were blocked by transfecting them with JNK-specific si-RNAs (Figs. 2A and 2B). Quantification of band intensity data showed that Cr(VI)-induced increases in cleaved Bid, caspase-3, and PARP were clearly reduced by either si-JNK1 or si-JNK2 transfection. Cr(VI) treatment increased early and late apoptotic population in JB6 cells. Approximately 3% of the cell population expressing high PI and low FITC signals, indicative of necrotic cells, was observed when the cells were exposed to 10μM $Na_2Cr_2O_7$. Under the same conditions, more than 60% of cell populations were apoptotic (Figs. 2C and 2D). The blockage of JNK expression by its specific si-RNA transfection attenuated the Cr(VI)-induced increase of Annexin V–positively stained cells. The protective effect of si-JNK1 and si-JNK2 transfection upon Cr(VI)-induced apoptosis was 15% ($p < 0.05$) and 21% ($p < 0.01$), respectively, which suggested the involvement of JNK-mediated signaling in Cr(VI)-mediated apoptosis. Cr(VI)-mediated reduction of $\Delta\Psi m$ was also inhibited by si-JNK transfection (Fig. 3), where the cells transfected with si-JNK2 recovered the content of $\Delta\Psi m$ in

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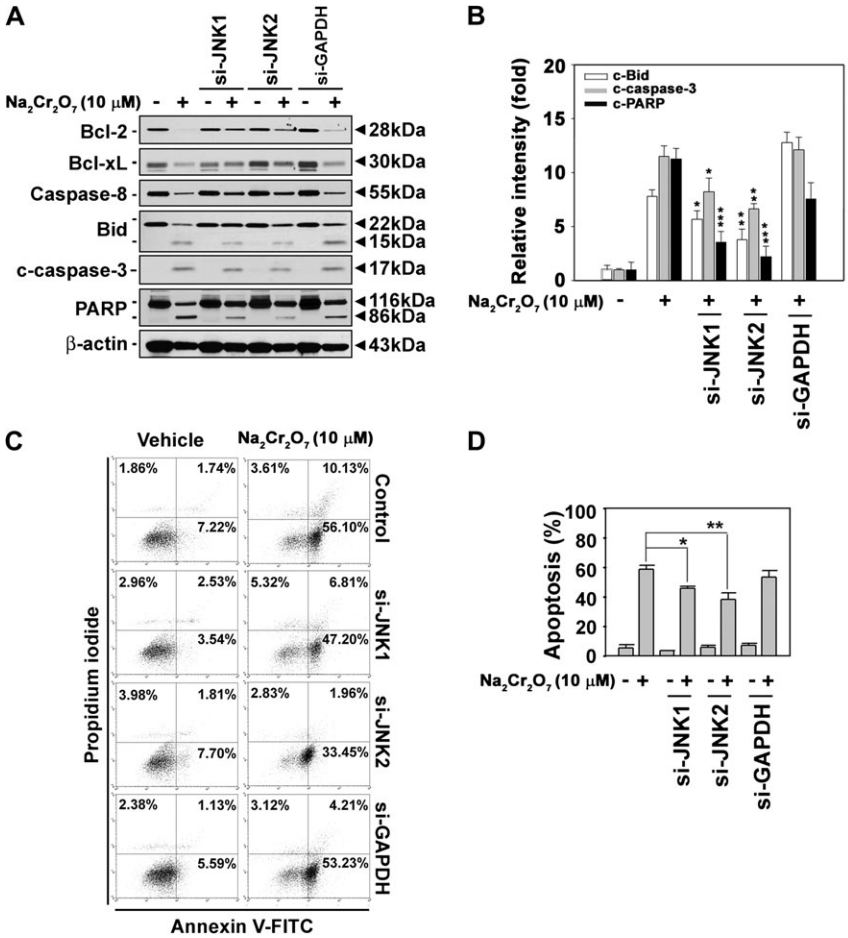

FIG. 2. JNK-mediated signaling plays a critical role in Cr(VI)-induced apoptosis in skin epidermal cells. JB6 cells were transfected with si-JNKs for 24 h. They were exposed to 10μM $Na_2Cr_2O_7$ for an additional 48 h and then processed by Western blot analysis (A) and Annexin V/PI staining (C). (B) The intensity of bands expressed as the mean ± SE relative to the control of triplicate experiments after normalizing the bands to β-actin. (D) The percentage of apoptotic populations was summed from early apoptotic cells (Annexin V^+/PI^-) and late apoptotic cells (Annexin V^+/PI^+). si-GAPDH and β-actin were used as control si-RNA and control protein, respectively. The results are shown as the mean ± SE of three separate experiments. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ versus the Cr(VI) treatment alone (ANOVA, Scheffe's test).

a significant level. This result indicated that JNK2 signaling more closely regulated mitochondrial stress than JNK1 in Cr(VI)-treated JB6 cells.

Cr(VI) Activates AP-1 in Skin Epidermal Cells

Because the transcription factor AP-1 and its subfamily c-Jun are well-known downstream mediators of JNK, we examined whether Cr(VI) is able to stimulate c-Jun phosphorylation and AP-1 activation using JB6 cells transfected with an AP-1-luciferase reporter plasmid (Li *et al.*, 1997). Cellular levels of c-Jun and p-c-Jun were augmented by treating the cells with Cr(VI) in a dose-dependent manner (Fig. 4A). Cr(VI) treatment also dramatically increased the luciferase activity specific for AP-1 in a dose- (Fig. 4B) and time-dependent manner (Fig. 4C), which suggested the involvement of AP-1–mediated signaling in cellular responses to Cr(VI).

c-Jun Mediates Survival Signal in Cr(VI)-Exposed Skin Epidermal Cells

We subsequently examined the role of c-Jun in Cr(VI)-exposed cells after si-c-Jun transfection. Figure 5 shows that the inhibition of c-Jun expression by si-RNA transfection facilitated the Cr(VI)-induced toxicity in the skin epidermal cells. In fact, the decrease in cell viability and the increase in Annexin V–positive cells, which had led in the Cr(VI)-treated cells, were accelerated significantly in the cells transfected with si-c-Jun. For example, when Cr(VI)-exposed cells were transfected with si-c-Jun, cell viability decreased to 11% compared with only Cr(VI)-treated cells (38%) (Fig. 5B). Apoptosis assay results also showed that the percentage of apoptotic cells, 53%, increased to 68% in si-c-Jun–transfected cells (Figs. 5C and 5D). Cr(VI)-induced decreases in $\Delta\Psi m$ accelerated in si-c-Jun–transfected

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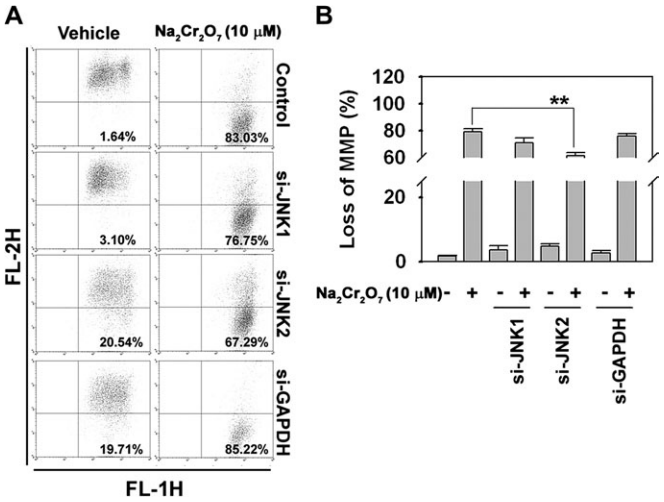

FIG. 3. Cr(VI) induced mitochondrial membrane potential ($\Delta\Psi m$) depolarization through JNK signaling pathway. (A) JB6 cells were treated with Cr(VI) for 48 h following transfection with si-JNKs. The cells were then incubated with 2μM JC-1 dye for 15 min, and the intensities of FL-1 and FL-2 fluorescence were measured by flow cytometry. (B) The percentage of depolarized populations was calculated from three independent experiments. $^{**}p < 0.01$ versus the Cr(VI) treatment alone (ANOVA, Scheffe's test).

cells when the cells were flow cytometrically analyzed after staining with JC-1 (Figs. 5E and 5F). These results suggested that c-Jun activation might have a protective effect against Cr(VI)-derived toxicity in skin epidermal cells.

c-Jun Regulates Intracellular $O_2^{\cdot-}$ Level in Cr(VI)-Exposed Cells

In order to understand the exact role of c-Jun in Cr(VI)-mediated cell death, we determined intracellular ROS levels using flow cytometry. Fluorescence intensity of dihydroethidium, which is specific for $O_2^{\cdot-}$, was increased in si-c-Jun–transfected cells as compared with the cells exposed to Cr(VI) alone (Figs. 6A and 6B). This result was supported by ESR measurements. These spin adducts showed an increased intensity cells transfected with si-c-Jun relative to the cells treated with Cr(VI) alone (Figs. 6C and 6D). In particular, the addition of SOD (50 U/ml) reduced the signal intensity of the spectrum, thereby suggesting that the increase in spin adduct intensity in the si-c-Jun–transfected cells was because of the generation of $O_2^{\cdot-}$. These results suggested that alteration of $O_2^{\cdot-}$ levels is dependent upon c-Jun in Cr(VI)-exposed cells. Western blot analysis confirmed the involvement of $O_2^{\cdot-}$ in Cr(VI)-mediated toxicity in skin epidermal cells. Antioxidant enzymes such as CAT, SOD1, SOD2, and GPX1 were slightly decreased in Cr(VI)-exposed cells (Fig. 7A). However, the Cr(VI)-mediated downregulation of SOD1 and SOD2 was transiently augmented by si-c-Jun transfection (Figs. 7B and 7C). These results suggested that c-Jun regulates SOD expression in Cr(VI)-exposed cells. It is possible that c-Jun survival signals in response to Cr(VI) are through the upregulation of cellular SOD expression. To further

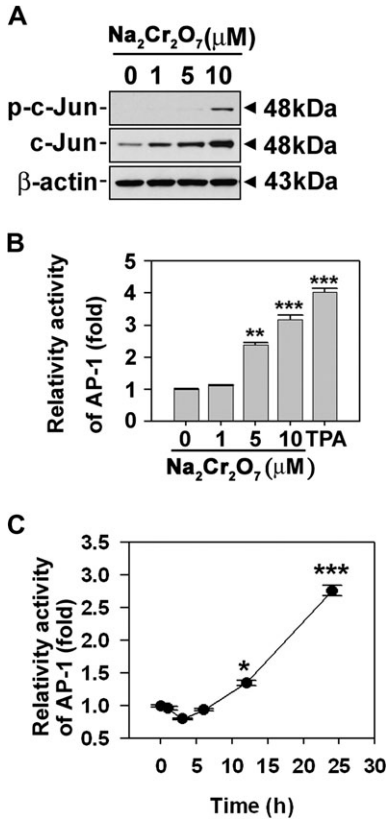

FIG. 4. Cr(VI) increased AP-1 activity in skin epidermal cells. (A) JB6 cells were incubated with $Na_2Cr_2O_7$ at the various concentrations (0–10μM) for 24 h. The levels of phosphorylated c-Jun and total c-Jun were detected by Western blot analysis. β-Actin was used as a control protein. (B) JB6 cells transfected with AP-1 promoter gene were exposed to the indicated concentrations (0–10μM) of $Na_2Cr_2O_7$ for 24 h. (C) Cells were exposed to 10μM $Na_2Cr_2O_7$ for various times (0–24 h). The AP-1 activity by luciferase assay, presented as relative AP-1 induction compared with the untreated control cells. 12-O-tetradecanoylphorbol-13-acetate (20 ng/ml) was used as positive control for AP-1 activity. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus the unexposed control values (ANOVA, Scheffe's test).

understand this phenomenon, we overexpressed SOD in the cells (Fig. 8). Overexpression of SOD1 or SOD2 caused Cr(VI)-induced cell death to decrease significantly ($p < 0.05$). This result indicated that expression of SOD regulated cell death in Cr(VI)-exposed JB6 cells. Moreover, both overexpressed SOD1 and SOD2 significantly attenuated si-c-Jun–mediated increases in cell death in Cr(VI)-exposed cells. This result further supported the hypothesis that c-Jun mediated cell survival signaling through regulation of SOD expression in Cr(VI)-exposed JB6 cells.

c-Jun Acts Upstream Effecter of p53 in Cr(VI)-Exposed Skin Epidermal Cells

We previously demonstrated that Cr(VI) caused apoptotic cell death in JB6 cells, where ROS generation and p53 upregulation play important roles (Son *et al.*, 2010a). We examined the effects of Cr(VI) on the phosphorylation of JNK and c-Jun in the presence of CAT. We also evaluated whether

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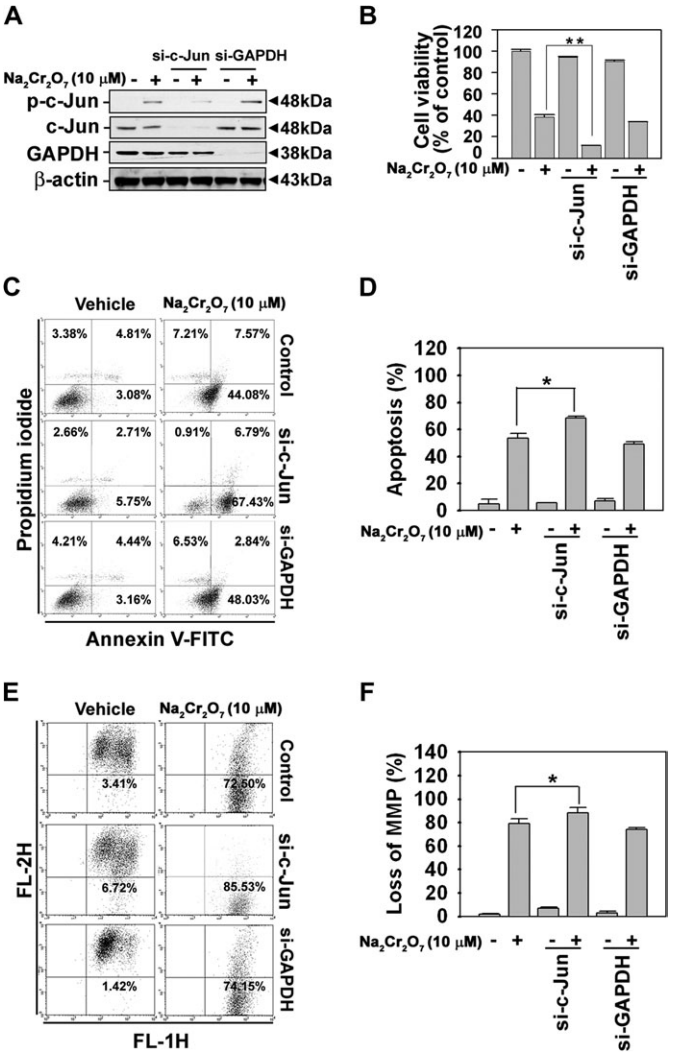

FIG. 5. Suppression of c-Jun expression by si-RNA transfection accelerated apoptotic cell death in Cr(VI)-exposed JB6 cells. JB6 cells were transfected with si-RNA specific to c-Jun for 24 h, after which they were exposed to 10μM $Na_2Cr_2O_7$ for additional 48 h and then processed for MTT assays (B), Annexin V/PI staining (C), or mitochondrial membrane potential (ΔΨm) (E) measurement. The knockdown in c-Jun levels was confirmed by Western blot analysis after 24 h Cr(VI) treatment (A). The percentages of apoptotic populations (D) and mitochondrial membrane–depolarized populations (F) were calculated from three independent experiments. si-GAPDH and β-actin were used as control si-RNA and control protein, respectively. $*p < 0.05$ and $**p < 0.01$ versus the Cr(VI) treatment alone (ANOVA, Scheffe's test).

there are the correlation between JNK-mediated signaling and p53 expression. Treating the cells with CAT (500 U/ml) diminished the Cr(VI)-mediated increases in p-c-Jun, p-JNKs, and p-p53 levels (Fig. 9A). These findings suggested that Cr(VI)-generated ROS participated in the activation of JNK/c-Jun–mediated signaling. The inhibition of JNK by transfection with specific si-RNA attenuated Cr(VI)-mediated induction of p-c-Jun levels, but there was increase in the p-p53 level (Fig. 9B). When the cells were transfected with si-p53, the levels of p-JNK and p-c-Jun were not affected regardless of the presence of Cr(VI) (Fig. 9C). si-c-Jun transfection did not change the phosphorylation state of JNK in Cr(VI)-exposed cells, although it did lead to a dramatic increase in p-p53 levels ($p < 0.001$) (Fig. 9D). These results suggested that the activation of c-Jun may play a role in cell survival by suppressing p53 expression as well as by regulating SOD expression in Cr(VI)-exposed cells.

DISCUSSION

We have previously reported that Cr(VI) induced apoptosis through ROS-mediated p53 activation in JB6 cells (Son *et al.*, 2010a). However, the p53 signaling pathway did not fully explain the mechanisms underlying Cr(VI)-induced apoptosis. The purpose of the present study is to determine whether there are other mediators, which participate in the induction of apoptosis in Cr(VI)-exposed cells. In this study, we have investigated specific mechanisms associated with JNK activation in response to ROS induced by Cr(VI) in JB6 cells. JNK exerts dual roles. It facilitates Cr(VI)-mediated apoptosis through the augmentation of caspase activation and mitochondrial stress. It also activates antiapoptotic c-Jun signaling in Cr(VI)-exposed cells, which leads to diminished p53 activation through upregulation of SOD expression.

Previous studies have demonstrated that Cr(VI) induced cell death by apoptosis (Bagchi *et al.*, 2001; Pritchard *et al.*, 2001; Singh *et al.*, 1998). In parallel, our current findings show that Cr(VI) induces phosphorylation of JNKs on skin epidermal cells in a dose- and time-dependent manner, which causes cell death through apoptotic mechanisms. Cr(VI)-induced cytotoxicity and apoptosis were decreased by si-JNK transfection (Figs. 1 and 2). This is because the increases in activated forms of cleaved caspase-3, Bid, and PARP by Cr(VI) were attenuated in si-JNK transfection (Figs. 2A and 2B). Moreover, mitochondrial membrane depolarization by Cr(VI) was significantly inhibited by si-JNK transfection (Fig. 3). However, JNK-mediated signaling did not play a major role in Cr(VI)-exposed cell death. This is because knockdown of JNKs with si-RNA prevented only 20–30% of Cr(VI)-induced apoptosis or loss of ΔΨm. Our previous study demonstrated that p53 signaling was also involved of Cr(VI)-induced apoptosis in JB6 cells (Son *et al.*, 2010a). Mitochondria play an important role in apoptosis induced by caspase-dependent or independent pathways, with Bcl-2 family proteins being critical for this induction. Our data show that decreases in Bcl-2 and Bcl-xL levels are attenuated by either si-JNK1 or si-JNK2 transfection (Fig. 2A). This indicates that activation of JNK by Cr(VI) causes downregulation of Bcl-2 and Bcl-xL proteins. Bcl-2 and Bcl-xL are the major antiapoptotic proteins integrated in the mitochondrial membrane. They prevent apoptosis through either heterodimerization with proapoptotic proteins or their direct pore-forming effects on the outer membrane of mitochondria (Gross *et al.*, 1999; Harris and Thompson, 2000; Nechushtan *et al.*, 2001).

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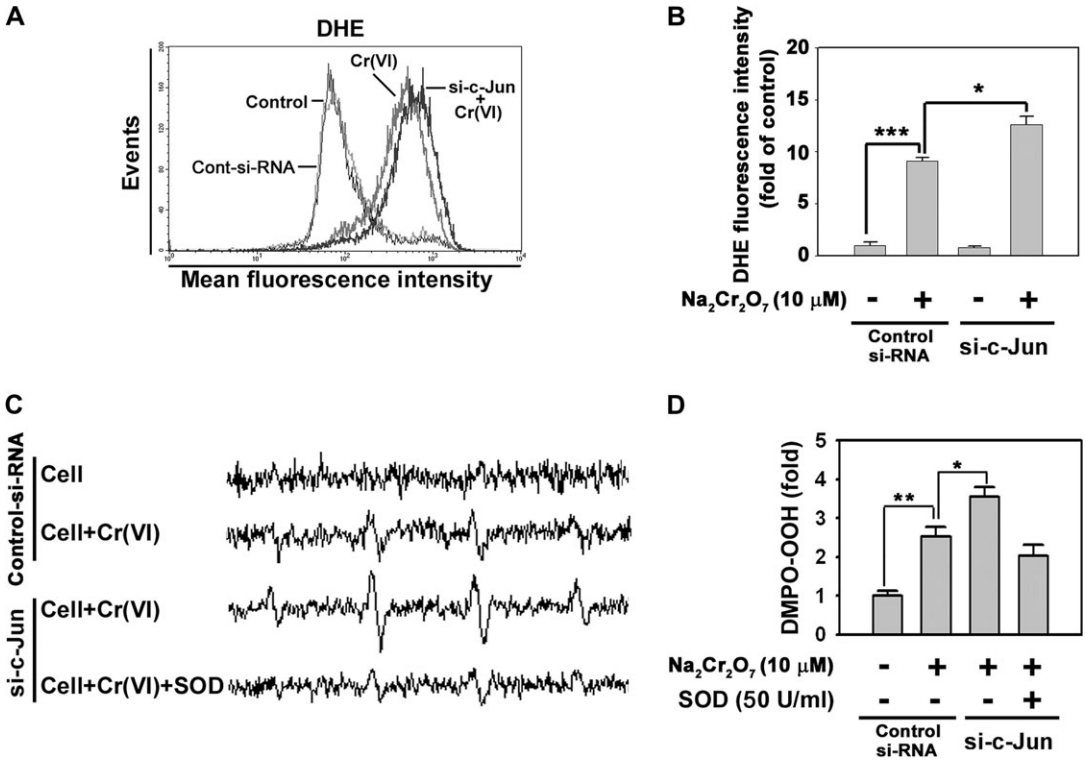

FIG. 6. Transfection with si-c-Jun increases intracellular $O_2^{\cdot-}$ levels in Cr(VI)-exposed cells. (A) JB6 cells were transfected with si-c-Jun for 24 h, were exposed to 10μM $Na_2Cr_2O_7$ for additional 24 h, and then processed for flow cytometry after treatment with 5μM DHE for 30 min. (B) The fluorescence intensity of DHE was expressed as the mean ± SE relative to the treatment control. (C) After transfection with si-c-Jun for 24 h, ESR spectra were recorded after the addition of 10μM $Na_2Cr_2O_7$ and 100mM DMPO to transfected cells (1×10^6) with or without SOD (50 U/ml). (D) The signal intensity of DMPO-OOH was expressed as the mean ± SE relative to the treatment control. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ versus the unexposed control si-RNA or the Cr(VI)-exposed control si-RNA (ANOVA, Scheffe's test). 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. DHE, dihydroethidium; DMPO, 5,5-dimethyl-1-pyrroline 1-oxide.

Although there is considerable evidence that c-Jun activation leads to apoptosis, c-Jun has diverse functions in stress response and apoptosis. For example, c-Jun is a positive regulator of cell growth in cultured fibroblast and hepatoblasts (Eferl *et al.*, 1999; Hilberg *et al.*, 1993; Johnson *et al.*, 1993). Moreover, the antiapoptotic function of c-Jun has been demonstrated in UV-induced cell death (Wisdom *et al.*, 1999) and is consistent with increased apoptosis in cultured hepatoblasts and erythroblasts lacking c-Jun (Eferl *et al.*, 1999). Therefore, the effect of c-Jun activation depends on the cell context and stimuli. In this study, we found that c-Jun acted in a prosurvival manner in Cr(VI)-exposed JB6 cells. Figure 5 shows that knocking down c-Jun expression by si-RNA transfection significantly enhances cytotoxicity and apoptosis in Cr(VI)-exposed cells. Moreover, decreases in $\Delta\Psi m$ are augmented by si-c-Jun transfection. These effects seem to be caused by c-Jun inhibition of p53 activity, as evidenced by the dramatic increase in phosphorylated p53 levels following si-c-Jun transfection (Fig. 9D). These findings demonstrate that c-Jun acts as an antiapoptotic factor rather than an effecter of apoptosis in Cr(VI)-exposed cells. c-Jun acts as a negative regulator of p53 expression by directly interacting with the p53 promoter (Schreiber *et al.*, 1999). However, our promoter assay shows that p53 luciferase activity is not significantly changed by si-c-Jun transfection (data not shown). This means that c-Jun regulates p53 activation indirectly. One possible mechanism by which c-Jun suppresses p53 is that c-Jun regulates SOD expression. ESR and fluorescence analyses have revealed that intracellular production of $O_2^{\cdot-}$ by Cr(VI) increases in si-c-Jun–transfected cells when compared with cells treated with Cr(VI) alone (Fig. 6). Our previous studies, as well as those by other groups, have shown that Cr(VI) leads to the generation of ROS such as ·OH, $O_2^{\cdot-}$, and H_2O_2 (Bagchi *et al.*, 2001; Ding and Shi, 2002; Son *et al.*, 2010a; Wang *et al.*, 2000). Cr(VI) directly generates ROS during its reduction and subsequent reaction with cellular small molecules, such as glutathione and H_2O_2 (Shi and Dalal, 1988, 1990a,b). Moreover, cells stimulated by Cr(VI) also generate ROS through nicotinamide adenine dinucleotide phosphate oxidase activation (Shi and Dalal, 1989, 1990d; Wang *et al.*, 2000). Otherwise, cells contain several ROS scavenging enzymes, such as CAT, GPX, and SOD. SOD is one of the

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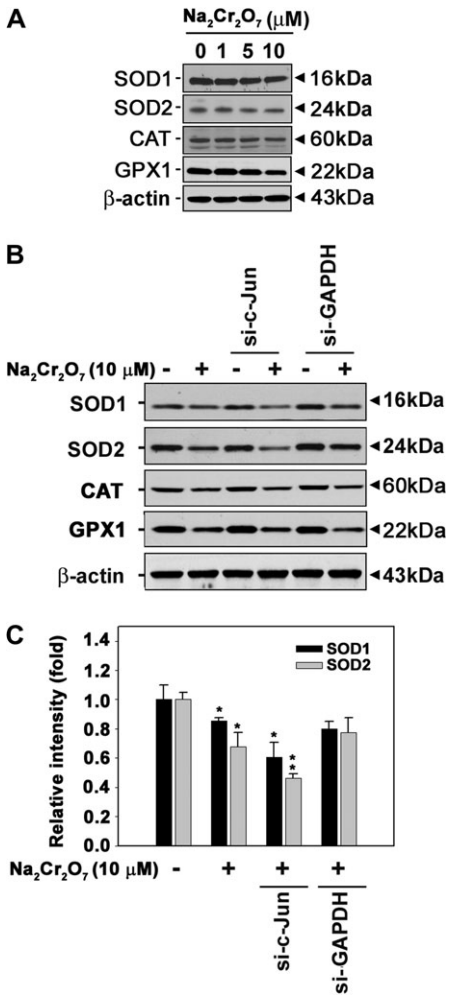

FIG. 7. Transfection with si-c-Jun attenuates SOD expression in Cr(VI)-exposed cells. (A) JB6 cells were incubated with $Na_2Cr_2O_7$ at various concentrations (0–10μM) for 24 h, and then the protein levels were detected by Western blot analysis. (B) Cells were transfected with si-c-Jun for 24 h, were exposed to 10μM $Na_2Cr_2O_7$ for additional 24 h, and then analyzed by Western blot. (C) The cellular protein levels of SOD1 or SOD2 were calculated using densitometry software and are expressed as the mean ± SE relative to the control after normalizing the bands to β-actin. Representative data were shown from triplicate experiments. $*p < 0.05$ and $**p < 0.01$versus the unexposed control. si-GAPDH and β-actin were used as control si-RNA and control protein, respectively.

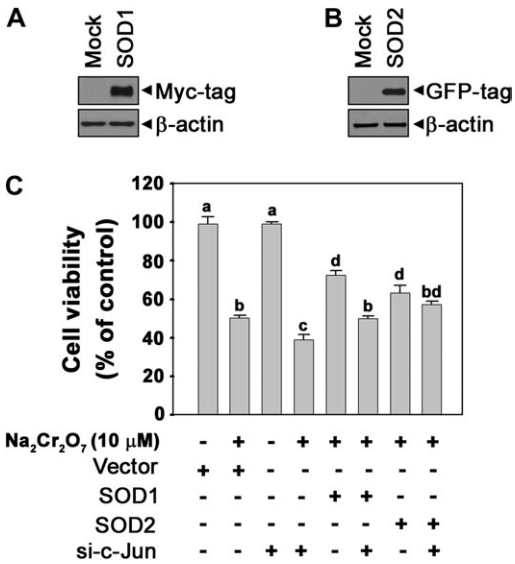

FIG. 8. SOD overexpression attenuates c-Jun knockdown–mediated increasing cell death in Cr(VI)-exposed cells. SOD1-Myc-DDK– and SOD2-EGFP–tagged plasmids were transfected into the JB6 cells. Overexpressed cellular levels of the proteins specific for SOD1 (A) or SOD2 (B) were confirmed by Western blotting. (C) SOD1 or SOD2 transformed JB6 cells were transfected with si-c-Jun for 24 h and incubated with $Na_2Cr_2O_7$ (10μM) for additional 48 h, after which an MTT assay was performed. The different superscripts represent significant differences ($p < 0.05$) between the groups according to Duncan's multiple range tests. β-actin and pCMV6 were used as a control protein and control vector, respectively.

primary antioxidant enzymes. In eukaryotic cells, there are two main forms of intracellular SOD: manganese-containing SOD(MnSOD) and copper/zinc-containing SOD(Cu/ZnSOD). MnSOD (SOD2) is located in the mitochondria matrix, whereas Cu/ZnSOD (SOD1) is found primarily in the cytoplasm. SOD regulates cell growth by changing the level of intracellular ROS (Liu *et al.*, 2002). In the present study, the expression of SOD1 and SOD2 are decreased by si-c-Jun transfection in Cr(VI)-exposed cells when compared with the cells treated with Cr(VI) alone (Fig. 7). However, the expression of CAT or GPX1 is not changed by si-c-Jun transfection. When cells overexpressed SOD1 or SOD2, Cr(VI)-induced cell death was attenuated (Fig. 8). This result shows that SOD reduces ROS production and prevents cell death in Cr(VI)-exposed JB6 cells. Moreover, the increase in cell death by c-Jun knockdown with si-RNA was prevented by both SOD1 and SOD2 overexpression. These results demonstrate that c-Jun regulates an intracellular balance in ROS levels through SOD expression in Cr(VI)-exposed JB6 cells. Although, overexpression of both SOD1 and SOD2 is effective, SOD2-overexpressed cells show more a pronounced protective effect than SOD1-overexpressed cells. This suggests that c-Jun more closely regulates SOD2 than SOD1 in Cr(VI)-exposed JB6 cells. Furthermore, the knockdown of c-Jun with si-RNA transfection increases $O_2^{\cdot-}$ (Fig. 6); this increase in ROS levels accelerates activation of p53 signaling (Fig. 9D). In addition, CAT downregulates Cr(VI)-induced activation of p53, JNK, and c-Jun (Fig. 9A). These results indicate that intracellular ROS generated by Cr(VI) is closely involved in Cr(VI)-induced apoptosis. Presumably, the signaling pathways of JNK, c-Jun, and p53 are simultaneously activated by Cr(VI) in JB6 cells.

In summary, this study demonstrates the dual effects of JNK signaling in Cr(VI)-exposed cells. JNK induces apoptosis through the caspase-dependent and mitochondria-mediated pathways, where the decreases in Bcl-2/Bcl-xL level, activation

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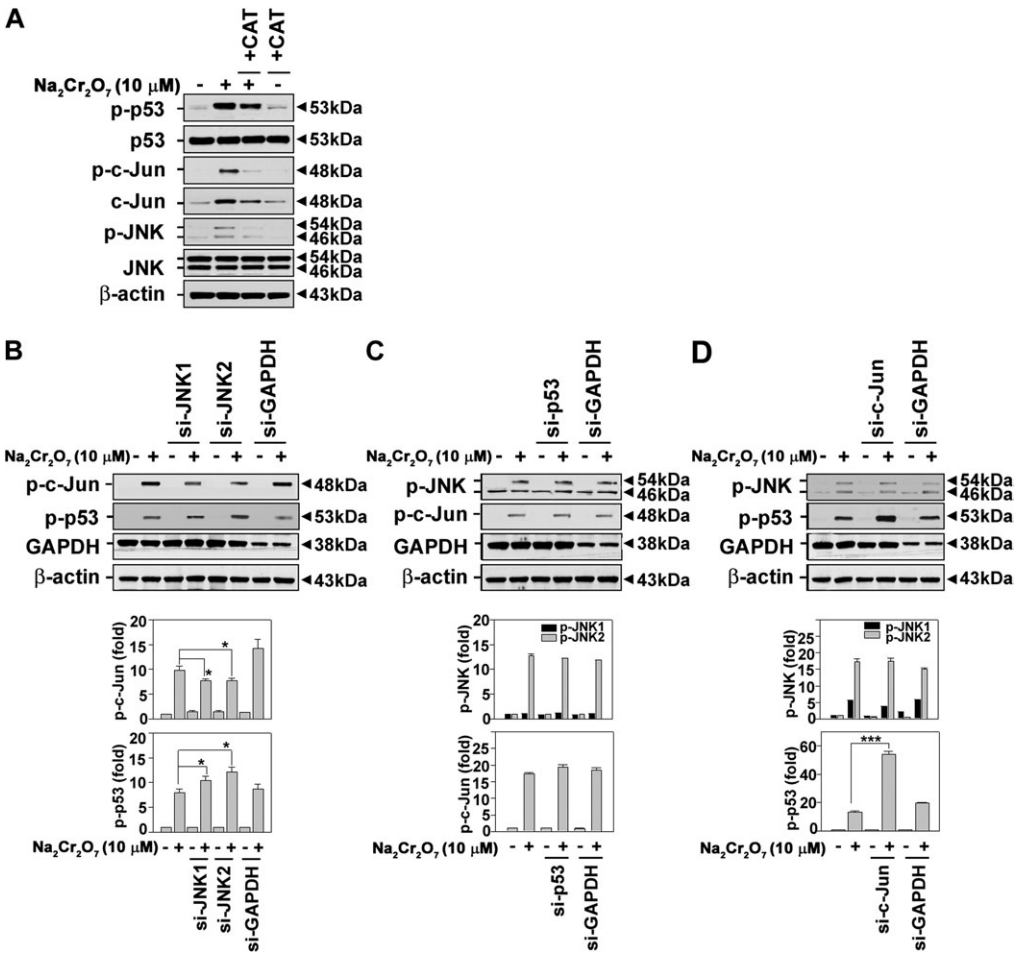

FIG. 9. Signal transduction pathways involved in Cr(VI)-induced apoptosis in skin epidermal cells. (A) Cells were treated with CAT (500 U/ml) in the presence or absence of $Na_2Cr_2O_7$ (10μM) for 24 h. Cells were transfected with si-RNA specific to JNK1, JNK2 (B), p53(C), and c-Jun (D) for 24 h, after which they were exposed to 10μM $Na_2Cr_2O_7$ for an additional 24 h, and protein levels were detected by immunoblot analysis. si-GAPDH and β-actin were used as control si-RNA and control protein, respectively. The intensity of bands was expressed as the mean ± SE relative to the control of triplicate experiments after normalizing the bands to β-actin. $*p < 0.05$ and $***p < 0.001$ versus the Cr(VI) treatment alone (ANOVA, Scheffe's test).

of Bid through caspase-8 signaling, and mitochondrial stress lead to caspase activation and PARP cleavage. c-Jun signaling induced by JNK is a survival mechanism against Cr(VI)-induced apoptosis through upregulation of SOD expression in skin epidermal cells (Fig. 10). This study provides a further understanding of cell survival or death mechanisms in heavy metal–exposed skin epidermal cells.

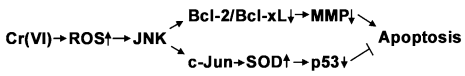

FIG. 10. The signaling pathways of JNK in Cr(VI)-exposed skin epidermal cells. Cr(VI) increases intracellular ROS level in JB6 cells. The ROS generated by Cr(VI) contribute to JNK activation. The activated JNK leads to mitochondrial dysfunction and apoptosis. c-Jun, which was activated by JNK, mediates survival signaling through inhibition of p53 activation because of upregulation of SOD expression in Cr(VI)-exposed JB6 cells.

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