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Roles of JNK, p38 and ERK mitogen-activated protein kinases in the growth inhibition and apoptosis induced by cadmium

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Cadmium (Cd), a human carcinogen, can induce apoptosis in various cell types. Three major mitogen-activated protein kinases (MAPKs), c-JUN N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), have been shown to regulate apoptosis. In this study we explore the ability of Cd to activate JNK, p38 and ERK, including their effects on Cd-mediated growth inhibition and apoptosis in a human non-small cell lung carcinoma cell line, CL3. The kinase activity of JNK was induced dose-dependently by 30–160 μM $CdCl_2$. High cytotoxic doses of Cd (130–160 μM) markedly activated p38, but low Cd doses did not. Conversely, the activities of ERK1 and ERK2 were decreased by low cytotoxic doses of Cd (≤80 μM) and moderately activated by high Cd doses. Low cytotoxic doses of Cd transiently activated JNK and simultaneously reduced ERK activity, whereas high cytotoxic doses of Cd persistently activated JNK and p38. PD98059, an inhibitor of ERK upstream activators MAPK kinase (MKK) 1 and MKK2, greatly enhanced cytotoxicity and apoptosis in cells treated with low Cd doses. In contrast, SB202190, an inhibitor of p38, decreased the cytotoxicity and apoptosis induced by high Cd doses. Transient expression of a dominant negative form of JNK1, but not that of JNK2, significantly increased the viability and prevented apoptosis of Cd-treated cells. However, expression of wild-type JNK1 did not affect viability and apoptosis of Cd-treated cells. Transfection of wild-type JNK2 or p38 enhanced apoptosis of cells exposed to low Cd doses but did not affect those exposed to high Cd doses. The JNK activity stimulated by low Cd doses was partially suppressed by expression of a dominant negative form of MKK7, but not a dominant negative form of MKK4, indicating that MKK7 is involved in JNK activation by Cd. Together, the results of this study suggest that JNK and p38 cooperatively participate in apoptosis induced by Cd and that the decreased ERK signal induced by low Cd doses contributes to growth inhibition or apoptosis.

Introduction

Three major mammalian mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), c-JUN N-terminal kinase (JNK) and p38 kinase, are regulated by distinct signal transduction pathways that control many aspects of mammalian cellular physiology, including cell growth, differentiation and apoptosis (1–4). MAPKs are activated by dual phosphorylation on Thr and Tyr within the motif Thr-Glu-Tyr (ERK), Thr-Pro-Tyr (JNK) or Thr-Gly-Tyr (p38) in subdomain VIII of the catalytic domain (1–4). This phosphorylation is mediated by a protein kinase cascade that consists of a MAPK kinase kinase that phosphorylates and activates one or more MAPK kinases (MKK) that, in turn, phosphorylate and activate each MAPK (1–4). JNK is known to be activated by MKK4 and MKK7 (4). Similarly, p38 is activated by MKK3, MKK6 and MKK4, whereas ERK is activated by MKK1 and MKK2 (4). Once activated, MAPKs regulate gene expression through phosphorylation of downstream transcriptional factors. For example, JNKs were originally identified by their ability to phosphorylate and activate the c-JUN N-terminal transactivation domain (5), whereas, ERK phosphorylates an inhibitory C-terminal DNA-binding domain of c-JUN (6,7).

In general, the ERK cascade is activated by growth factors and is critical for cell proliferation (8). Conversely, the JNK and p38 pathways are stimulated by genotoxic agents and cytokines mediating the stress response, growth arrest and apoptosis (9–14). However, controversial evidence has indicated that more complex roles of these MAPK pathways exist to transmit other ultimately distinct cellular effects in different cell lineages. For example, ERK promotes differentiation in neuronal cells, T cells and muscle cells (15,16), in contrast to transmiting mitogenic signals in fibroblasts. On the other hand, stress-activated JNK and p38 have been associated with cell survival (17,18), anti-cytotoxicity (19,20), anti-apoptosis (21,22), transformation (23–25), proliferation (26) and invasion (27). The complexities of the physiological roles of MAPKs may be due partially to the duration of MAPK activities differentially regulating genes in various cell types. While transient ERK activation leads to proliferation, persistent activation mediates growth arrest or differentiation signals (28–30). In contrast, transient JNK and p38 induction could provide a survival signal, whereas persistent activation induces apoptosis (31,32).

Cd is a ubiquitous environmental toxicant that has been evaluated as a human carcinogen (33). The main sources of human exposure to Cd are tobacco smoke, food and industrial pollution. Cd is absorbed by inhalation and ingestion and has a biological half-life >25 years. For cigarette smoke, up to 60% of Cd deposited in the lung is absorbed (34). In experimental animals Cd compounds have been shown to induce tumors in lung, testes, prostate, the hematopoietic system and at injection sites (33,35). Cd compounds have been shown to induce morphological transformations, chromosomal aberrations and gene mutations in cultured mammalian cells (36–40). Cd is highly reactive with sulfhydryl groups of proteins and can substitute for zinc in certain enzymes (41–43). Cd has been shown to induce mRNA levels of several genes such as

Abbreviations: ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; GST, glutathione *S*-transferase; JNK, c-JUN N-terminal kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MKP, MAPK phosphatase; MTT, 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline.

c-*jun* (44), c-*myc* (44), c-*fos* (45), metallothionein genes (46) and heme oxygenase (47,48). Cd has been reported to activate p38 and ERK in 9L rat brain tumor cells (49) and to activate JNK in LLC-PK1 porcine renal epithelial cells (50). Moreover, Cd promotes apoptosis in various cells (49,51–53), but the roles of MAPKs in Cd-mediated growth arrest and apoptosis have not been explored. In this study we have investigated the ability of Cd to induce AP-1 DNA-binding activity, c-*jun* expression and the activities of JNK, p38 and ERK in a human lung adenocarcinoma cell line, CL3. The roles of these three MAPKs in growth arrest and apoptosis induced by Cd were investigated using PD98059, a specific inhibitor of MKK1 and MKK2 (ERK upstream kinases), and SB202190, a specific inhibitor of p38, or by transient transfection assay to block activation of JNK. The results have suggested that p38 activated persistently by high cytotoxic doses of Cd induces an apoptotic signal, while decreased ERK activity induced by low cytotoxic doses of Cd contributes to growth arrest and apoptosis. The transfection results have suggested that JNK1 is more important than JNK2 in growth inhibition and apoptosis of Cd-treated cells. Additionally, activation of JNK by Cd is mediated through an MKK7-dependent and MKK4-independent pathway.

Materials and methods

Cell culture

Cell line CL3, established from a non-small cell lung carcinoma tumor of a 60-year-old male patient in Taiwan (54), was provided by Dr P.C. Yang (Department of Internal Medicine and Clinical Pathology, National Taiwan University Hospital, Taipei, Taiwan). Cells were cultured in RPMI 1640 medium (Gibco Life Technologies, Grand Island, NY) supplemented with sodium bicarbonate (2.2% w/v), L-glutamine (0.03% w/v), penicillin (100 U/ml), streptomycin (100 μg/ml) and fetal calf serum (10%). Cells were maintained at 37°C in a humidified incubator containing 5% CO_2 in air.

Transfection

Plasmids containing dominant negative forms of JNK1 (pCMV-JNK1-APF; JNK1DN) or JNK2 (pSR-JNK2-APF; JNK2DN), MKK4 (JNKK[K116R]; MKK4KR) or wild-type forms of JNK1 and JNK2 were kindly provided by Dr M. Karin (University of California, San Diego, CA) (5,55,56). Plasmids containing a dominant negative form of MKK7 (MKK7A) and the wild-type form of p38 were gifts from Dr J. Han (The Scripps Research Institute, La Jolla, CA) (57,58). Cells (4×10^5) were plated in a 60 mm dish 1 day before transfection. Plasmids (5 μg) were transfected into CL3 cells by calcium phosphate co-precipitation. After incubation for 6 h, the cells were washed with phosphate-buffered saline (PBS) and cultured in complete medium for 24 h, followed by maintenance in serum-free medium for 16–18 h. The cells were then subjected to Cd treatment.

Treatment

Cells in exponential growth were plated at a density of 1×10^6 cells/100 mm dish and cultured in serum-free medium for 16–18 h before Cd treatment. Cadmium chloride (Merck, Darmstadt, Germany) was dissolved in MilliQ purified water (Millipore, Bedford, MA). Cells were treated with $CdCl_2$ for 0.5–12 h in serum-free medium. In experiments to determine the effects of PD98059 (Calbiochem, San Diego, CA) and SB202190 (Calbiochem) on Cd-induced MAPKs, cell growth inhibition, cytotoxicity and apoptosis, cells were treated with these kinase inhibitors for 1 h and then co-exposed to $CdCl_2$ for 3 h. At the end of treatment, the drug-containing medium was removed and the cells were washed twice with PBS.

Cell growth

After treatment, the cells were cultured in medium containing 10% serum for 0–3 days before trypsinization. A portion of cells were mixed with 0.4% trypan blue (Gibco Life Technologies) for 15 min and the number of unstained cells were determined using a hemocytometer.

Viability assay

Cells were seeded in 96-well plates at a density of 5×10^3 cells/well and cultured in serum-free medium for 16–18 h before $CdCl_2$ treatment. The cells were treated with $CdCl_2$ for 3 h in the presence or absence of kinase inhibitors. After treatment, the cells were cultured in medium containing 10% serum for 24 h. The cells were treated with 500 μg/ml 3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical Co., St Louis, MO) and cultured for 3 h in a CO_2 incubator. Cells having functional mitochondrial succinate dehydrogenase can convert MTT to formazan that generates a blue color when dissolved in dimethyl sulfoxide (59). The intensity was measured using a reader for enzyme-linked immunosorbent assay and an absorption wavelength of 565 nm.

Apoptosis

The annexin V–fluorescein isothiocyanate (FITC) binding assay was adopted for determination of apoptosis. Briefly, 2×10^5 cells were washed twice with PBS and suspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$). The cells were stained with annexin V–FITC (1 ng/μl) (Medical and Biological Laboratories Ltd, Japan) and propidium iodide (5 ng/μl) for 15 min in the dark. The stained cells were analyzed using flow cytometry and the CellQuest program (Becton Dickinson). Apoptotic cells were detected as those stained with annexin V–FITC but not with propidium iodide.

Whole cell extract preparation

Cells were lysed in a buffer containing 20 mM HEPES at pH 7.6, 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM Na_3VO_4, 50 mM NaF, 0.5 μg/ml leupeptin, 1 μg/ml aprotinin, and 100 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride. The cell lysate was rotated at 4°C for 30 min, centrifuged at 10 000 r.p.m. for 10 min and the precipitates discarded. Protein concentrations were determined by the BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Kinase assay

The JNK activity assay was performed using glutathione *S*-transferase (GST)–cJun(1–79) (kindly provided by Dr M.Karin) as substrate according to the procedure described by Hibi *et al*. (5) with modifications. Briefly, 50–200 μg of protein prepared from cell extracts were mixed with 5 μg of GST–cJun (1–79) and 20 μl of glutathione–Sepharose 4B suspension (Amersham Pharmacia Biotech, Arlington Heights, IL) at 4°C for 3 h then centrifuged. After three washes in HEPES binding buffer (20 mM HEPES, pH 7.6, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA and 0.05% Triton X-100) and one wash in kinase buffer (20 mM HEPES, pH 7.6, 20 mM $MgCl_2$, 2 mM DTT and 0.1 mM Na_3VO_4), the pelleted beads were resuspended in 30 μl of kinase buffer containing 20 μM ATP and 2 μCi [γ-^{32}P]ATP (6000 Ci/mmol; Amersham, Pharmacia Biotech). The reaction was performed at room temperature for 30 min and terminated by washing with HEPES binding buffer. Phosphorylated proteins were eluted with 30 μl of 1.5× Laemmli sample buffer, boiled for 5 min and resolved on 10% SDS–polyacrylamide gels. The relative radioactivity was quantitated using a computing densitometer equipped with the ImagQuant analysis program (Molecular Dynamics, Sunnyvale, CA). The in-gel kinase assay was conducted by resolving 50 μg of total proteins on a 10% SDS–polyacrylamide gel containing 40 μg/ml GST–cJun(1–79) fusion protein as described by Hibi *et al*. (5).

Western blot analysis

Cellular protein (20–50 μg) was loaded onto 10% SDS–polyacrylamide gels. The protein bands were then transferred electrophoretically to PVDF membranes (Micron Separations Inc., Westborough, MA). Membranes were probed with primary antibody, followed by a horseradish peroxidase-conjugated second antibody (Bio-Rad, Hercules, CA). Phospho-specific antibodies for p38 (9211) and ERK (9101) were purchased from New England BioLabs Inc. (Beverly, MA). Anti-JNK1 antibody (G151-666) was purchased from Pharmingen (San Diego, CA). Anti-ERK2 (C-14) and anti-p38 (C-20) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibody reactions were detected using the enhanced chemiluminescence detection procedure according to the manufacturer's recommendations (Amersham, Pharmacia Biotech).

Isolation and analysis of RNA

Total RNA (15 μg), isolated by a guanidium isothiocyanate/phenol/chloroform extraction procedure (60), was subjected to electrophoresis in a 1% agarose–2.2 M formaldehyde gel, transferred to nylon membranes and hybridized to ^{32}P-labeled DNA probes prepared by *redi*prime random primer labeling (Amersham Pharmacia Biotech) with a specific activity of $>1.5\times10^8$ c.p.m.. Hybridization was performed overnight at 42°C in a solution containing 50% formamide, 6× SSC (1× SSC = 180 mM NaCl, 10 mM sodium citrate, pH 8.0), 5× Denhardt's reagent, 0.5% SDS and 100 μg/ml salmon sperm DNA. Membranes were washed to a stringency of 0.1× SSC, 0.1% SDS for 10 min at 65°C and exposed to X-ray films.

Electrophoretic mobility shift assay

Nuclear protein was extracted as described by Andrews and Faller (61). Binding reactions were performed at room temperature with 20 μl of a mixture containing 6 μg nuclear protein, 10 mM Tris–HCl, pH 7.6, 3% glycerol,

50 mM KCl, 2.25 mM $MgCl_2$, 0.125% NP-40, 2 μg bovine serum albumin and 1 μg poly(dI·dC). After incubation for 20 min, 1 ng ^{32}P-labeled oligonucleotides (~20 000 c.p.m.) was added and incubated at room temperature for another 30 min. The DNA–protein complexes were resolved on 6% non-denaturing polyacrylamide gels at 4°C, dried and visualized by autoradiography. For competition experiments, a 100-fold excess of unlabeled oligonucleotides was added to the reaction mixture before adding the labeled probe. The sequences of the oligonucleotides used, specific for the AP-1 binding site and that containing a point mutation, were 5′-CGATCGCAT-GACTCACTCAAT-3′ and 5′-CGATCGCAgGACTCACTCAAT-3′, respectively (the AP-1 binding site is underlined and the point mutation is in lower case; only one strand is shown).

Results

Growth rate and viability of Cd-treated CL3 cells

The effects of Cd on the cellular growth rate and viability of human lung adenocarcinoma CL3 cells were examined in order to understand Cd cytotoxicity. Cells in exponential growth were cultured in serum-free medium for 18 h before they were exposed to various $CdCl_2$ concentrations for 3 h. The cells were maintained in culture in complete medium for 0–3 days and the numbers of viable cells were determined by the trypan blue exclusion assay. Figure 1A shows that the growth rates of CL3 cells treated with 30 or 60 μM Cd were not significantly different from untreated cells, whereas 130 or 160 μM Cd markedly inhibited cell growth ($P < 0.01$). Although the number of viable cells was decreased by 80 μM Cd ($P < 0.05$), these cells could proliferate at a normal rate 2–3 days after treatment (Figure 1A). The relative survival of cells treated with Cd was determined by the MTT assay. Approximately 70 and 5% of cells survived after exposure to 80 and 160 μM Cd, respectively (Figure 1B).

Induction of c-jun expression and AP-1 DNA-binding ability by Cd in CL3 cells

CL3 cells were treated with $CdCl_2$ in serum-free medium for 3 h and subjected to northern blot analysis to examine the ability of Cd to induce c-*jun* mRNA. Figure 2A shows that Cd (50–150 μM) markedly induced c-*jun* mRNA above the background level. The induced c-*jun* expression may subsequently enhance the amount of AP-1 transcription factor and its DNA-binding activity (2). The nuclear proteins of Cd-treated and untreated CL3 cells were then extracted to examine AP-1 DNA-binding activity using the electrophoretic mobility shift assay. The nuclear extract obtained from untreated control cells had little AP-1 DNA-binding activity, whereas it was markedly induced by 100–200 μM Cd in CL3 cells (Figure 2B). Competition experiments showed that the induced AP-1 transcription factor interacts with a specific DNA AP-1-binding sequence but does not interact with an AP-1 site containing a point mutation (Figure 2B). These results clearly show that Cd in the absence of serum can induce c-*jun* mRNA levels and AP-1 DNA-binding activity in CL3 cells.

Cd alters the activities of JNK, p38 and ERK in CL3 cells

The transcription of c-*jun* is regulated by binding of a c-JUN/ATF-2 heterodimer to the promoter (2). Both c-JUN and ATF-2 are activated by the JNK pathway; ATF-2 is also activated by the p38 pathway (2,4). Moreover, AP-1 activation is correlated with the ERK pathway (2). We therefore examined the ability of Cd to induce JNK, p38 and ERK activities in serum-starved CL3 cells. JNK activity in CL3 cells was determined using [γ-^{32}P]ATP and a specific JNK substrate, GST–cJUN(1–79). Activation of p38 and ERK were determined using specific antibodies recognizing the dual phosphorylation sites of the catalytic domain. CL3 cells in exponential growth were serum starved for 18 h prior to $CdCl_2$ treatment for 3 h. Western blot analyses showed that the amounts of JNK, p38 and ERK proteins in Cd-treated cells were the same as in untreated cells (Figure 3), whereas activation of these MAPKs was markedly altered by Cd in a dose-dependent manner (Figure 3). JNK activity was increased 2-fold by 30 μM Cd and dramatically increased by 60–160 μM Cd (Figure 3A). Cd concentrations of 130–160 μM markedly activated p38 but lower Cd concentrations (15–80 μM) did not increase it (Figure 3B). In contrast, ERK activity was decreased by 15–80 μM Cd and increased 4-fold at higher Cd concentrations (130–160 μM) (Figure 3C). In-gel kinase assays showed that both JNK1 and JNK2 activities were induced in cells exposed to 100 μM Cd for 3 h (Figure 3D). The induced kinase activities were higher than that induced by 40 J/m^2 UVC, a well-known JNK activator (5; Figure 3D).

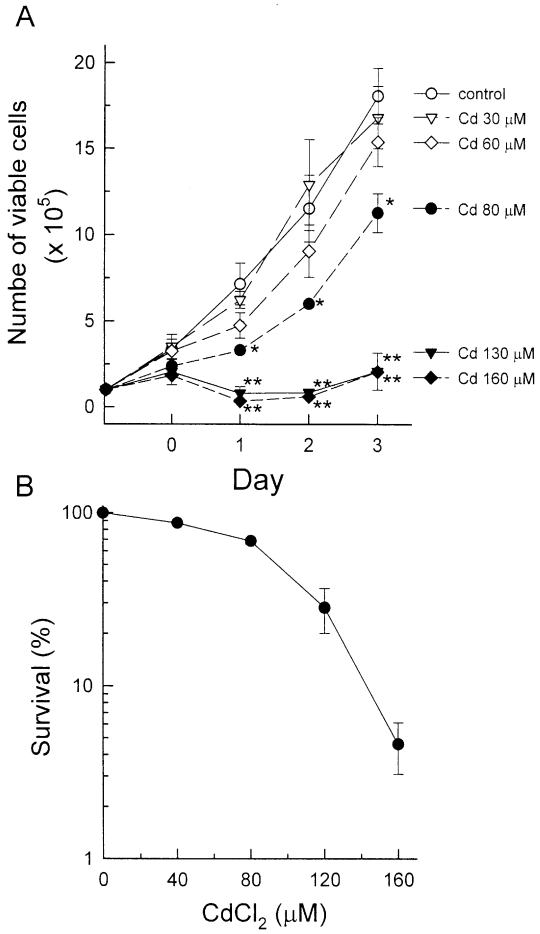

Fig. 1. Growth rate and viability of $CdCl_2$-treated CL3 cells. (**A**) Cells (1×10^5) in exponential growth were plated and cultured for 24 h in complete medium followed by serum starvation for 18 h. The cells were then treated with Cd in serum-free medium for 3 h, washed with PBS and cultured in medium containing 10% serum for 0–3 days. Cells were trypsinized, stained with trypan blue and the numbers of viable cells were determined with a hemocytometer. (**B**) The viability of cells was determined by MTT assay 1 day after exposure to Cd. Results were obtained from three or four experiments and the bar represents SEM. *$P < 0.05$ and **$P < 0.01$ using Student's *t*-test for the comparison between untreated and Cd-treated cells.

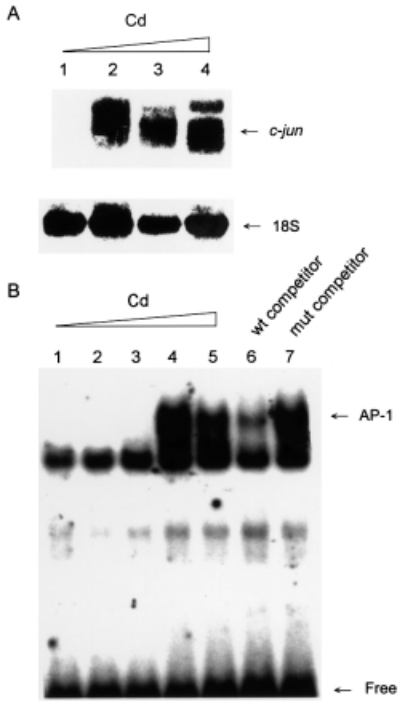

Fig. 2. Induction of c-*jun* expression and AP-1 DNA-binding activity by $CdCl_2$ in CL3 cells. Cells were treated with Cd in serum-free medium for 3 h. Total RNA isolation and assay of AP-1 DNA-binding activity were performed as described in Materials and methods. (**A**) Northern blot analysis shows that the expression of c-*jun* mRNA was induced by 50, 100 and 150 μM Cd (lanes 2–4). Expression of 18S rRNA was analyzed to serve as an internal control. (**B**) Electrophoretic mobility shift assay shows that the nuclear extract obtained from untreated control cells (lane 1) or cells treated with 10–50 μM Cd (lanes 2 and 3) had little AP-1 DNA-binding activity, whereas it was markedly induced by 100–200 μM Cd (lanes 4 and 5). Competition experiments were carried out by adding a 100-fold excess of non-isotope-labeled wild-type AP-1 oligonucleotide (lane 6) or oligonucleotides having a point mutation (lane 7) to the reaction mixture containing the nuclear sample of lane 4 before adding the isotope-labeled probe. Autoradiographs shown are from a representative experiment, which was repeated three times with comparable results.

Figure 4 shows the activities of these MAPKs in a time course Cd treatment. CL3 cells in exponential growth were serum starved for 18 h and exposed to 80 μM Cd for 0.5–12 h. The maximum activity of JNK induced by 80 μM Cd in CL3 cells was observed after a 3 h treatment, and decreased after a longer period (6–12 h) of Cd exposure (Figure 4). In contrast, p38 activity increased when the cells were exposed to 80 μM Cd for 6–12 h in a time-dependent manner (Figure 4). ERK activity abated during Cd exposure for 0.5–3 h and increased above the control levels by 6–12 h treatment (Figure 4).

To further investigate the stability of Cd-activated JNK and p38, Cd-treated CL3 cells were washed with PBS, cultured in Cd-free medium for another 0, 1 or 8 h and then assayed for JNK and p38 activities. Figure 5 shows that the JNK activity induced by 80 μM Cd markedly decreased with increased incubation time post-treatment, whereas JNK activity induced by 150 μM Cd remained at high levels when the metal was removed from the medium for 8 h (Figure 5). The results suggest that Cd at low doses transiently activates JNK, whereas high doses of Cd persistently activate it. High doses of Cd also persistently activate p38, whereas low Cd doses do not increase it (Figure 5).

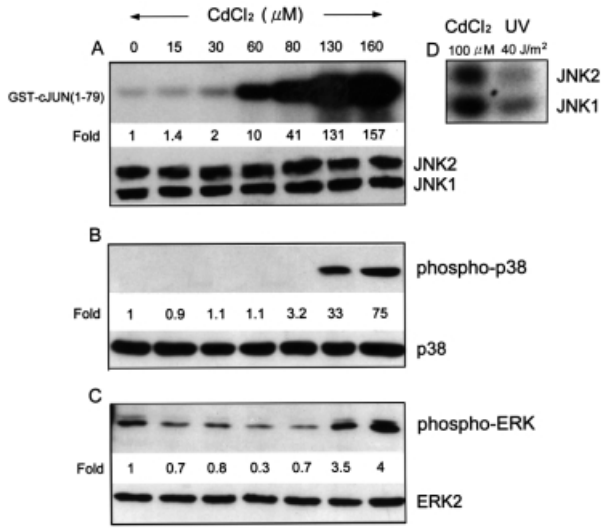

Fig. 3. Dose-dependent activation of JNK and p38 and alteration in ERK activity by $CdCl_2$ in CL3 cells. (**A**) Cells were treated with Cd (0–160 μM) in serum-free medium for 3 h. The proteins (200 μg) prepared from the whole cell extract were assayed for JNK activity using GST–cJUN(1–79) as substrate. The same cell extracts obtained from Cd-treated CL3 cells were examined for amounts of dual phosphorylated p38 and ERK using phospho-specific p38 (**B**) and phospho-specific p44/p42 MAPK antibodies (**C**), respectively. The lower panels in (A)–(C) represent western blot analyses of JNK, p38 and ERK2 protein levels. The relative intensity was quantitated using a computing densitometer equipped with the ImagQuant analysis program. The numbers in the middle of (A)–(C) indicate the relative activities of kinases calculated from the average of three experiments. (**D**) In-gel kinase analysis of JNK in Cd-treated (100 μM, 3 h) or UV-irradiated (40 J/m^2, 254 nm) CL3 cells. The whole cell extract of UV-irradiated cells were isolated 30 min after treatment. The fluence rate of UV was measured with a UVX radiometer (UVP Inc.).

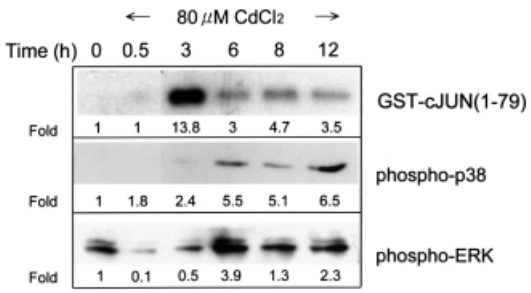

Fig. 4. Time-dependent activation of JNK and p38 and reduction of ERK by low doses of $CdCl_2$ in CL3 cells. Cells were treated with 80 μM Cd in serum-free medium for 0.5–12 h. Proteins (50 μg) prepared from whole cell extract were assayed for JNK activity using GST–cJUN(1–79). The ERK and p38 activities were determined by phospho-specific antibodies. The relative activities of kinases shown are calculated from the average of three experiments.

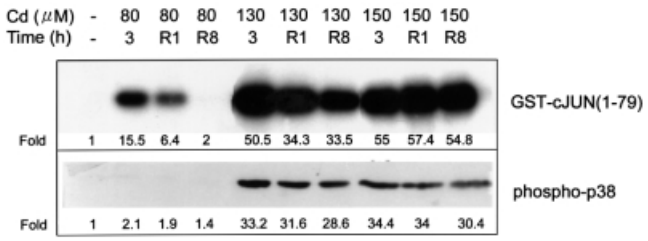

Fig. 5. Duration of the activation of JNK and p38 by $CdCl_2$ in CL3 cells. Cells were treated with Cd (80–150 μM) in serum-free medium for 3 h, washed with PBS and cultured continually in RPMI 1640 medium containing 10% serum for 0, 1 and 8 h. The relative activities of kinases shown are calculated from the average of three experiments.

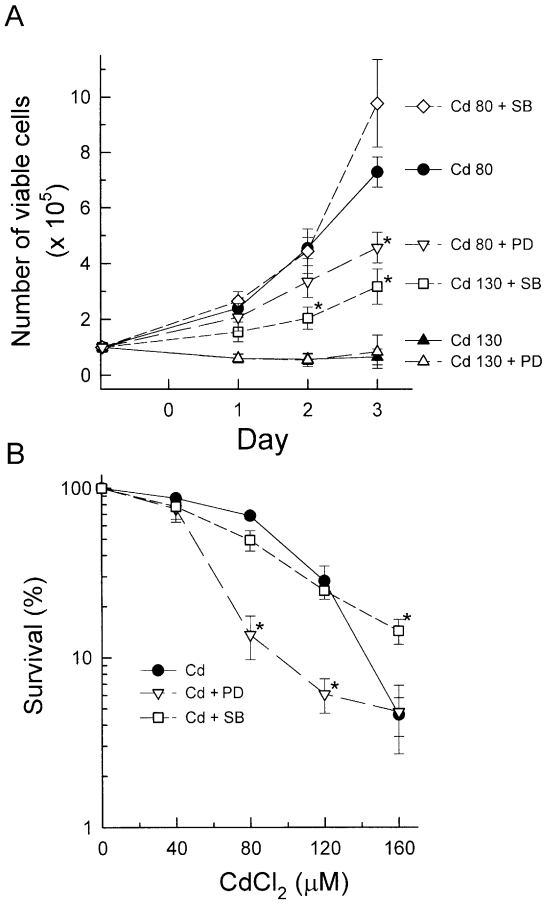

Fig. 6. Effects of SB202190 and PD98059 on the growth rate and viability of $CdCl_2$-treated CL3 cells. PD98059 (50 μM) or SB202190 (10 μM) was added to cells 1 h before Cd treatment for 3 h. The growth rate (**A**) and viability (**B**) were determined as described in Figure 1. Results were obtained from between three or five experiments and bars represent SEMs. $^*P < 0.01$ using Student's *t*-test for comparison between Cd-treated cells and those exposed to the same doses of Cd in the presence of PD98059 or SB202190.

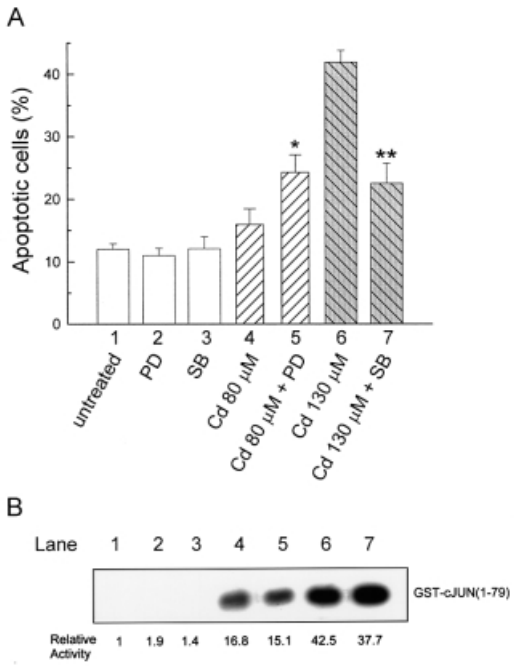

Fig. 7. SB202190 attenuates, whereas PD98059 potentiates $CdCl_2$-induced apoptosis in CL3 cells. (**A**) PD98059 (50 μM) or SB202190 (10 μM) was added to cells 1 h before Cd treatment for 3 h. Cells undergoing apoptosis were examined at 8 h post-exposure to Cd using the annexin V–FITC binding assay. Results were obtained from five or six experiments and bars represent SEMs. $^*P < 0.05$ and $^{**}P < 0.01$ using Student's *t*-test for comparison between Cd-treated cells and those exposed to the same doses of Cd in the presence of PD98059 or SB202190. (**B**) SB202190 or PD98059 did not alter Cd-induced JNK activity. Cells were exposed to Cd in the presence or absence of kinase inhibitors as in (A) and the whole cell extract was isolated for determination of JNK activity. Each lane corresponds to the treatment as labeled in (A). The relative activities of kinases shown are calculated from the average of three experiments.

Opposing roles of ERK and p38 in Cd-induced cytotoxicity and apoptosis

To inspect the roles of ERK and p38 signaling pathways in Cd cytotoxicity, CL3 cells were exposed to Cd in the presence of the kinase inhibitors PD98059 and SB202190, specific for MKK1/2 (ERK upstream kinases) and p38, respectively. The numbers of viable cells were determined using the trypan blue exclusion assay. Figure 6A shows that PD98059 (50 μM) decreased the viability of cells exposed to 80 μM Cd ($P < 0.01$, day 3), whereas SB202190 (10 μM) potentiated the numbers of viable cells in populations treated with 130 μM Cd ($P < 0.01$, days 2 and 3). The effects of these kinase inhibitors on Cd-induced cytotoxicity were also determined by MTT assay. The results confirmed that PD98059 greatly enhanced Cd cytotoxicity ($P < 0.01$, 80 and 120 μM), while SB202190 inhibited the cytotoxicity induced by high doses of Cd ($P < 0.01$, 160 μM; Figure 6B). These results indicate that ERK and p38 have opposing roles in Cd-mediated cytotoxicity.

The annexin V–FITC binding assay was adopted to determine the role of apoptosis in Cd-mediated cell death. Figure 7A shows that Cd induced significant numbers of apoptotic cells in a dose-dependent manner, ~40% of cells undergoing apoptosis 8 h post-exposure to 130 μM Cd for 3 h. Co-administrating 10 μM SB202190 with 130 μM Cd markedly reduced the frequency of apoptotic cells ($P < 0.01$; Figure 7A). In contrast, 50 μM PD98059 co-administration enhanced apoptotic cell induction by 80 μM Cd ($P < 0.05$; Figure 7A). Additionally, co-administrating PD98059 or SB202190 with Cd did not affect the JNK activity induced by Cd (Figure 7B). PD98059 or SB202190 alone neither increased the number of apoptotic cells nor JNK activity above untreated control cells. The results suggest that the p38 signal pathway, persistently activated by high doses of Cd, participates in cytotoxicity and apoptosis. Also, decreased ERK signaling by low doses of Cd contributes to growth arrest or apoptosis.

Roles of JNK1 and JNK2 in cell growth and apoptosis of Cd-treated CL3 cells

Recent evidence indicates that individual JNK isoforms may transmit distinct signals to regulate protective and cell death-inducing pathways. For example, expression of JNK1 but not JNK2 restores the ability of yeast lacking HOG1 (a p38 homolog) to grow in hypertonic medium (62). Expression of dominant negative JNK1 (JNK1DN) but not dominant negative JNK2 (JNK2DN) markedly increased the resistance of small cell lung cancer cells to UV-induced apoptosis (63). Therefore, we investigated the effect of Cd-induced JNK1 and JNK2 on cell growth and apoptosis by transfection of CL3 cells with vectors containing JNK1DN, JNK2DN or the wild-type forms of JNK1 and JNK2. Cells transfected with 5 μg of vectors were allowed 2 days for expression and then treated with Cd

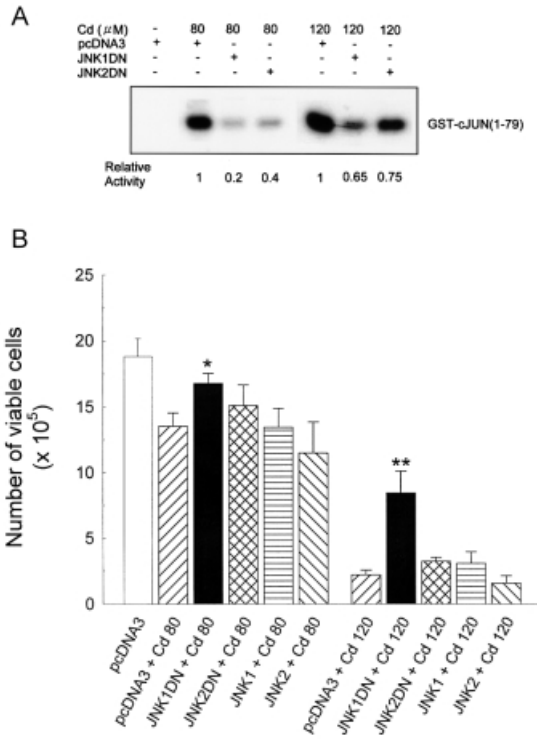

Fig. 8. Transient transfection assay to determine the effects of JNK1 and JNK2 on the growth rate of $CdCl_2$-treated CL3 cells. Cells (4×10^5) were plated in a 60 mm dish 1 day before transfection. Vectors containing JNK1DN, JNK2DN, JNK1 and JNK2 (5 μg each) were co-precipitated with calcium phosphate and transfected into CL3 cells. After incubation for 6 h, the cells were washed in PBS and resuspended in complete medium for 24 h, followed by sustenance in serum-free medium for 16–18 h. The cells were then treated with 80 or 120 μM Cd for 3 h. (**A**) The kinase activity of JNK was determined immediately after Cd treatment. The relative activities of kinases shown are calculated from the average of three experiments. (**B**) The numbers of viable cells were determined 24 h after Cd treatment by the trypan blue exclusion assay. Data were obtained from three or four independent experiments and bars represent SEMs. The statistical analysis compares cells containing JNK1DN treated with Cd and those containing pcDNA3 exposed to the same dose of Cd using Student's *t*-test (*$P < 0.05$ and **$P < 0.01$).

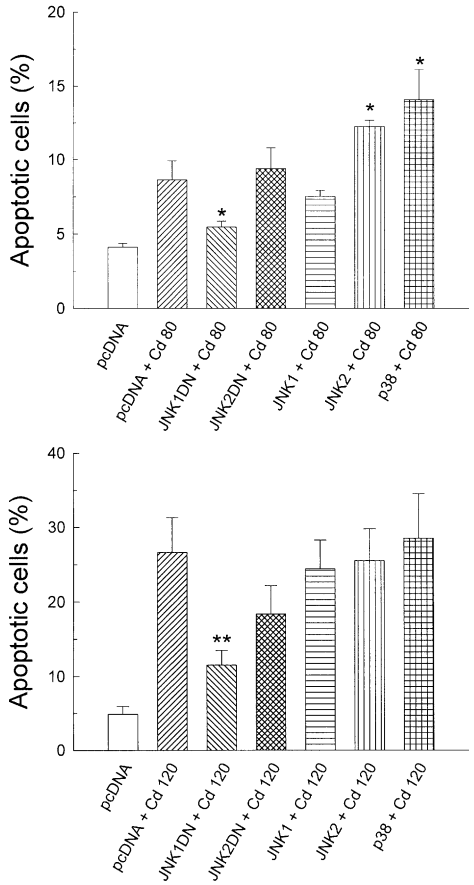

Fig. 9. Effects of JNK1, JNK2 and p38 on apoptosis induced by $CdCl_2$ in CL3 cells. Transient transfection and expression of JNK1DN, JNK2DN, JNK1, JNK2 or p38 (5 μg each) in cells followed by Cd treatment were performed as described in Figure 8. Cells undergoing apoptosis were examined 24 and 8 h post-exposure to 80 and 120 μM Cd, respectively, using the annexin V–FITC binding assay. Results were obtained from between four or six experiments and bars represent SEMs. The statistical analyses compare cells containing JNK1DN, JNK2 or p38 treated with Cd and those containing pcDNA3 exposed to the same dose of Cd using Student's *t*-test (*$P < 0.05$ and **$P < 0.01$).

for 3 h in serum-free medium. Figure 8A shows that transient expression of JNK1DN and JNK2DN blocked the activity of JNK induced by Cd. JNK1DN was more efficient than JNK2DN in arresting activation of JNK by Cd, suggesting that Cd activated JNK1 more than JNK2. Transient expression of JNK1DN or JNK2DN did not affect the activities of p38 and ERK induced by Cd (data not shown). The effects of Cd-activated JNK1 and JNK2 on cell growth inhibition and apoptosis were determined using the trypan blue exclusion and annexin V–FITC binding assays. The numbers of viable cells in Cd-treated populations were significantly increased by expression of JNK1DN in CL3 cells (80 μM Cd, $P < 0.05$; 120 μM Cd, $P < 0.01$), whereas JNK2DN had no effect (Figure 8B). Similarly, JNK1DN significantly abated Cd-induced apoptosis but JNK2DN did not (Figure 9). Conversely, transfection of wild-type JNK1 into CL3 cells influenced neither growth inhibition nor apoptosis caused by Cd (Figures 8B and 9). In contrast, transfection of wild-type JNK2 or p38 could enhance apoptosis induced by low Cd doses in CL3 cells ($P < 0.05$; Figure 9). These results suggest that JNK1 in combination with JNK2 and p38 transmit a signal for Cd-induced growth inhibition and apoptosis.

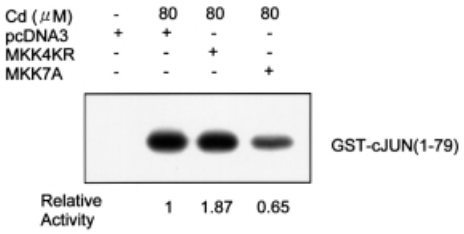

Fig. 10. Effects of MKK7 and MKK4 on activation of JNK by $CdCl_2$ in CL3 cells. Cells were transfected with kinase-defective MKK7A or MKK4KR (5 μg each) and exposed to Cd as described in Figure 8. The relative activities of JNK shown are calculated from the average of nine experiments.

MKK7 but not MKK4 is involved in Cd induction of JNK activity

Both MKK4 and MKK7 are reported to be upstream activators of JNKs by various stimuli (64–68). To determine the effect of these MKKs on Cd-induced JNK activity, we transfected CL3 cells with a dominant negative form of MKK4 (MKK4KR) or MKK7 (MKK7A). The results showed that 35% of the JNK activity stimulated by Cd (80 μM) was suppressed by expression of MKK7A in CL3 cells (Figure 10). In contrast,

expression of MKK4KR enhanced Cd-induced JNK activity ~2-fold (Figure 10). The ability of MKK7A to suppress Cd-induced JNK activity, however, was not observed in cells treated with 130 μM Cd (data not shown).

Discussion

In the present study we have examined the ability of $CdCl_2$ to activate JNK, p38 and ERK and the effects of these MAPKs on Cd-mediated growth arrest and apoptosis in CL3 human lung adenocarcinoma cells. Cd at low cytotoxic doses transiently activated JNK and concomitantly reduced ERK activity, whereas high cytotoxic doses of Cd persistently activated JNK and p38. Co-administration of Cd with specific inhibitors to block ERK or p38 signaling showed that these pathways have opposing effects on the growth arrest and apoptosis induced by Cd. Inhibition of ERK activity by PD98059 accelerated growth inhibition and apoptosis induced by low Cd doses, suggesting that ERK is involved in cell proliferation and anti-apoptosis. Inhibition of p38 activity by SB202190, however, enhanced cell viability and prevented apoptosis induced by high Cd doses, suggesting that persistently activated p38 participates in apoptosis. Transient expression of JNK1DN, but not JNK2DN, in CL3 cells markedly increased cell viability and prevented apoptosis in Cd-treated populations. On the other hand, transfection of wild-type JNK2 or p38, but not JNK1, could enhance apoptosis induced by low Cd doses in CL3 cells. Low Cd doses did not activate p38 and may activate JNK1 more than JNK2. This would explain in part why transfection of wild-type p38 or JNK2 enhanced apoptosis when the cells were exposed to low Cd doses. The results shown here suggest that Cd may sequentially activate JNK1, JNK2 and p38 in a dose-dependent manner to cooperatively induce apoptosis. Decreased ERK activity may combine with increased JNK activity to transmit a signal temporally causing growth arrest by low Cd doses in CL3 cells.

Separate signaling modules may provide specific responses of MAPK pathways to different stimuli. For example, MKK1 and MKK2 specifically activate the ERK group, MKK3 and MKK6 specifically activate p38 and MKK4 activates both p38 and JNK (2). Recently, MKK7/JNKK2 was identified as phosphorylating and activating JNK, but not p38 or ERK (67,68). We have demonstrated that JNK activity stimulated by low Cd doses is suppressed by expression of a dominant negative form of MKK7. In contrast, expression of a dominant negative form of MKK4 moderately enhances Cd-induced JNK activity. It has been shown that JNK activation by various stimuli is mediated differentially through MKK4 and MKK7. For example, tumor necrosis factor α and interleukin 1 activate MKK7 much more than MKK4, suggesting that JNK activation by these stimuli is primarily through MKK7; anisomycin and UV activate MKK4 more than MKK7 (64,66). The results of this study suggest that JNK activation by Cd is at least in part by activation of its upstream activator MKK7 and is independent of MKK4 in CL3 cells. Nevertheless, kinase-defective MKK7A could not completely block JNK activation by low Cd doses and did not suppress its activation by high Cd doses. It is possible that Cd may induce JNK upstream activators other than MKK7 and MKK4; the existence of such an uncharacterized JNK upstream activator has been observed in cells exposed to a hyperosmolar environment (66). Alternatively, Cd may destroy a negative control pathway which blocks specific MAPK phosphatases (MKPs) (69) or inhibitors (70,71).

MKPs are encoded by immediate early genes and induced in response to environmental stresses and growth factor stimulation to selectively down-regulate MAPK activities (69). For example, the cytosolic M3/M6 MKP is highly specific for JNK and p38 inactivation, while the association of MKP-3 with ERK in the cytosol markedly stimulates ERK dephosphorylation and inactivation (72–74). Activation of the JNK pathway in NIH 3T3 fibroblasts could result in MKP-1 gene expression and ERK inactivation, suggesting the existence of cross-talk between the JNK and ERK signaling pathways (75). However, Cd did not affect the total amounts of MKP-1 in CL3 cells (S.-M.Chuang, unpublished data), suggesting that MKP-1 may not be involved in the regulation of MAPK activities in Cd-treated cells. Whether Cd affects expression of MKPs deserves further investigation.

Proteins that interact and suppress MAPKs may also block these signaling pathways. For example, GST π was recently identified as associated with JNK and suppressed its kinase activity (71). The anti-apoptotic effect of thioredoxin is reported to be associated with inhibition of ASK1, a MAPK kinase kinase that activates JNK and p38 (70). Both GST and thioredoxin play important roles in detoxification of reactive oxygen species and the balance of cellular redox levels (76,77). The genotoxicity of Cd is correlated with intracellular reactive oxygen species (39,78,79). Cd may down-regulate the functions of GST π and thioredoxin, thereby elevating JNK and p38 activities. Moreover, the scaffold or adaptor proteins that interact with multiple components of a specific signal cascade can influence activation and function of distinct kinases. For example, the adaptor protein JIP-1 (JNK interacting protein 1), which specifically binds to JNK, is required for JNK activation (80), however, JIP-1 overexpression causes cytoplasmic retention of JNK and subsequently suppresses JNK activity to phosphorylate nuclear transcriptional factors (80,81). It will be important to explore the mechanism by which Cd activation of JNK and p38 is mediated by its capability to block cellular MAPK inhibitors.

We have shown here that the expression of c-*jun* mRNA was markedly increased in CL3 cells treated with 50–150 μM Cd for 3 h. JNK was also activated by the same Cd doses. These findings agree with the notion that JNK phosphorylates c-JUN/ATF-2 which binds to the promoter of c-*jun* (2). The Cd-induced c-*jun* mRNA would enhance c-JUN levels, thereby increasing AP-1 DNA-binding activity (2). However, AP-1 DNA-binding activity was induced at somewhat higher doses of Cd. The failure to observe enhanced AP-1 binding activity at the low Cd doses that were able to induce JNK kinase activity and c-*jun* expression may be due in part to the fact that the specific AP-1-binding site used here was the promoter sequence of *gadd*153. Furthermore, activated JNK is not always correlated with increased levels of c-*jun* transcripts and AP-1 binding activity (82,83).

The cytotoxicity of Cd is highly associated with the levels of intracellular metallothionein, which sequesters Cd and alleviates its toxicity (84). Cd can induce expression of metallothionein in CL3 cells as determined by northern blot analysis (I.-C.Wang, unpublished data). This may explain why CL3 cells are more resistant to Cd than those such as Chinese hamster ovary K1 cells that are deficient for metallothionein (39). Nevertheless, $CdCl_2$ (4 μM, 3 h, ~30% cytotoxicity) can also markedly activate JNK in Chinese hamster ovary K1 cells (S.-M.Chuang, unpublished data), suggesting that metallothionein is not required for JNK activation by Cd. Furthermore,

$CdCl_2$ (30 μM, 3 h, ~30% cytotoxicity) significantly activates JNK in HFW normal diploid human fibroblasts (S.-M.Chuang, unpublished data), indicating that the ability of Cd to activate JNK is not restricted to CL3 lung adenocarcinoma cells.

Cd is classified as a human carcinogen (33). However, several reports have shown an ability of Cd to suppress tumor growth and progression in rodent lung and liver (85,86). Cd has been shown to induce apoptosis in various cells (49,51–53). The relationship between apoptosis and tumor suppression by Cd is not clear (86). Our finding that the JNK and p38 signal pathways induced by Cd participate in growth arrest and apoptosis of human lung adenocarcinoma cells suggests a potential role of these MAPKs in Cd-mediated tumor suppression.

In summary, we have demonstrated here that low cytotoxic doses of Cd activate JNK, reduce ERK and do not affect p38, producing convergent signals transiently inhibiting cell growth, whereas high cytotoxic doses of Cd persistently activate JNK and p38 to induce apoptosis. JNK activation by Cd is mediated through a MKK7-dependent and MKK4-independent pathway. Further investigation into the mechanisms by which Cd alters the functions of specific regulators of JNK and p38 would greatly help to elucidate the role of MAPK components in Cd-mediated apoptosis and carcinogenesis.

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