Sustained Activation of JNK-p38 MAP Kinase Pathways in Response to Cisplatin Leads to Fas Ligand Induction and Cell Death in Ovarian Carcinoma Cells

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Running title: CDDP-induced JNK/p38 mediates FasL expression and apoptosis
SUMMARY

The efficacy of cisplatin in cancer chemotherapy is limited by the development of resistance. Although the molecular mechanisms involved in chemoresistance are poorly understood, cellular response to cisplatin is known to involve activation of mitogen-activated protein kinase (MAPK) and other signal-transduction pathways. An understanding of early signal-transduction events in the response to cisplatin could be valuable for improving the efficacy of cancer therapy. We compared cisplatin-induced activation of three MAPKs—JNK, p38 and ERK—in a cisplatin-sensitive human ovarian-carcinoma cell line (2008) and its -resistant subclone (2008C13). The JNK and p38 pathways were activated differentially in response to cisplatin, with the cisplatin-sensitive cells showing prolonged (8-12 h) activation and the cisplatin-resistant cells showing only transient (1-3 h) activation of JNK and p38. In the sensitive cells, inhibition of cisplatin-induced JNK and p38 activation blocked cisplatin-induced apoptosis; persistent activation of JNK resulted in hyperphosphorylation of the c-Jun transcription factor, which in turn stimulated the transcription of an immediate downstream target, the death inducer Fas ligand (FasL). Sequestration of FasL by incubation with a neutralizing anti-FasL antibody inhibited cisplatin-induced apoptosis. In contrast, chemoresistance in the 2008C13 cells was associated with a failure in upregulation of FasL. Moreover, in these cells, selective stimulation of the JNK/p38 MAP kinase pathways by adenovirus-mediated delivery of recombinant M KK7 or M KK3 led to sensitization to apoptosis through reactivating FasL expression. Thus the JNK > c-Jun > FasL > Fas pathway plays an important role in mediating cisplatin-induced apoptosis in ovarian cancer cells, and the duration of JNK activation is critical in determining whether cells survive or undergo apoptosis.
INTRODUCTION

Cisplatin [cis-diaminedichloroplatinum(II)] (CDDP)\(^1\) is a platinum-based compound that forms intra- and interstrand adducts with DNA (1,2). CDDP has a broad spectrum of antitumor activity and is widely used in the treatment of solid tumors (3). However, one of the major limitations in its efficacy is that many tumors either are inherently resistant or acquire resistance after an initial response (1-4). The molecular mechanisms that underlie this chemoresistance are largely unknown. Possible mechanisms of acquired resistance to CDDP include decreased platinum accumulation, elevated drug inactivation by metallothionine and glutathione, and enhanced DNA repair activity (5,6). Increased expression of antiapoptotic genes and mutations in the intrinsic apoptosis pathway may contribute to the inability of cells to detect DNA damage or induce apoptosis (1,7-9).

Because of the reactivity of CDDP and the complexity of the cellular response to DNA damage, CDDP-induced apoptosis signaling likely involves several pathways. Elucidation of the details of these signaling pathways is important since it may explain why tumor cells exposed to cisplatin often lose sensitivity to this agent and become resistant to apoptotic signals.

Genotoxic stress induces multiple signal transduction pathways, among which are the mitogen-activated protein kinase (MAPK) pathways. These pathways are parallel cascades of structurally related serine/threonine kinases that play pivotal roles in transducing various extracellular signals to the nucleus. The MAPK signaling cascades regulate a variety of cellular activities, including cell growth, differentiation, survival, and death (10,11). In mammals, MAPKs are divided into three major groups—ERKs, JNKs or SAPKs, and p38, based on their degree of homology, biological activities and phosphorylation motifs (12). Even though these signaling systems are built from evolutionarily related protein kinases, they produce distinct biological responses. The biological effects of MAPK signaling are executed by phosphorylation of downstream substrates, most notably a number of signal-responsive
transcription factors. The broad range of these substrates indicates that MAPKs have pivotal roles in cellular signal transduction and suggests that the extent and duration of MAPK activation play key roles in controlling cell functions.

The ERK pathway, which is induced in response to mitogenic stimuli such as peptide growth factors, cytokines, and phorbol esters, involves ERK1 and ERK2 (ERK1/2), the participation of Raf-1 and Ras oncoproteins, and the activation of MEK1/2 (12). Once activated, ERK phosphorylates several substrates, including Elk-1 (13). The ERK pathway plays a major role in regulating cell proliferation and differentiation (12) and provides a protective effect against apoptosis (14). The signaling cascades involving JNK and p38, on the other hand, are key mediators of stress signals and seem to be responsible mainly for protective responses, stress-dependent apoptosis, and inflammatory responses. These cascades can be stimulated by various stresses, such as UV- or gamma-irradiation, osmotic stress, heat shock, proinflammatory cytokines such as tumor necrosis factor-alpha or interleukin-1β, and chemotherapeutic drugs (10,12).

To understand the molecular basis for the failure of CDDP-based chemotherapy, we compared the cellular response of the human ovarian carcinoma cell line 2008 and its resistant subclone 2008C13 (15) after treatment with a platinum-based anticancer agent. We found that differences in the duration of the activation of MAPK pathways correlated with CDDP-induced apoptosis. A strong, sustained activation of both pathways seemed to be a required priming step for CDDP-induced apoptosis; this activation of both JNK and p38 MAPK in CDDP-sensitive cells correlated with upregulation of the Fas ligand (FasL), an immediate downstream target of JNK, and was accompanied by the induction of caspase activity and apoptosis. Cisplatin’s failure to elicit such a response in the resistant variant indicated that impaired FasL expression could contribute to the development of chemoresistance. Reduction of cisplatin-induced apoptosis by the expression of a dominant-negative c-Jun that lacked JNK phosphoacceptor sites or by the use of either a small drug inhibitor of p38/JNK or a neutralizing anti-FasL antibodies further underlined the
critical role of c-Jun-dependent FasL-expression signaling in the induction of apoptosis by genotoxic agents.

EXPERIMENTAL PROCEDURES

Reagents—Cisplatin (Platinol-AQ cisplatin injection) was obtained from Bristol Laboratories (Princeton, NJ). Polyclonal antibodies to p38, phospho-p38 (T180/Y182), ATF-2, phospho-ATF-2 (T71), JNK, phospho-JNK (T183/Y185), c-Jun, phospho-c-Jun (S73), Erk, and phospho-Erk (T202/Y204), were purchased from Cell Signaling (Beverly, MA). The monoclonal anti-JNK1 antibody clone 333.8, anti-human PARP antibody, anti-caspase-8 antibody, monoclonal anti-human cytochrome c, the neutralizing anti-human FasL antibody NOK-2 and an isotope-matched control antibody were obtained from BD-Pharmingen (San Diego, CA). The anti-Fas monoclonal antibody (CH-11) was purchased from Medical & Biological Laboratories (Watertown, MA). The anti-capase-3/CPP32 antibody was purchased from Transduction Laboratories (Lexington, KY). Anti-beta-actin monoclonal antibodies were obtained from Sigma Chemical Co. (St. Louis, MO). The caspase inhibitor zVAD-fmk and the JNK and p38 kinase inhibitor SB202190 were purchased from Alexis Biochemicals (San Diego, CA). CDDP-sensitive (2008) and CDDP-resistant (2008C13) ovarian cancer cells were kindly provided by Drs. S. B. Howell (University of California San Diego, La Jolla, CA), S. G. Chaney (University of North Carolina, Chapel Hill, NC), and Z. H. Siddik (M. D. Anderson Cancer Center, Houston, TX). The 2008 cell line, established from a patient with serous cystadenocarcinoma of the ovary, and its resistant subclone 2008C13, derived from 2008 by in vitro exposure to CDDP, have been characterized by Howell and coworkers (15,16). Wild-type (wt) and c-jun−/−3T3 fibroblasts were a gift from Drs. E. F. Wagner (Research Institute for Molecular Pathology, Vienna, Austria) and M. Karin (University of California San Diego, La Jolla, CA).
Cell Culture and Adenoviral Infection—The CDDP-sensitive human ovarian cell line 2008 and its resistant variant 2008C13 were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Life Technologies, Inc.), and 1% streptomycin-penicillin. The 3T3 and 3T3 c-jun<sup>−/−</sup> cells were cultured in Dulbecco’s modified Eagle’s medium supplemented as described above. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Recombinant adenovirus vectors expressing green fluorescent protein (Ad-GFP) and activated mutants of MKK7 and MKK3 (Ad-MKK7D and Ad-MKK3bE) were constructed as previously described (17). Cells were infected with adenoviruses at a multiplicity of infection of 50 plaque-forming units (pfu)/cell for 5 h and then incubated for another 30 h to allow expression of the protein of interest, as described elsewhere (17).

Immunoblot Analysis—Cells in log-phase growth were treated or not treated with CDDP at 20 µM (2008 and 2008C13 cells) or 100 µM (3T3 and 3T3 c-jun<sup>−/−</sup> cells) for 1 h, after which they were washed and fresh medium was added. At various times after CDDP exposure (1 min and 1, 3, 5, 8, 12 h), the cells were collected and lysed in lysis buffer (25 mM Hepes, pH 7.7, 400 mM NaCl, 0.5% Triton X-100, 1.5 mM MgCl<sub>2</sub>, 2 mM EDTA, 2 mM dithiothreitol, and 0.1 mM phenylmethyl-sulfonyl fluoride; protease inhibitors [10 µg/ml leupeptin, 2 µg/ml peptstatin, 50 µg/ml antipain, 2 µg/ml aprotinin, 20 µg/ml chymostatin, benzamidine 2 µg/ml] and phosphatase inhibitors [50 mM NaF, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, and 20 mM β-glycerophosphate]). For PARP and caspase immunoblots, cell lysates were prepared using the RIPA lysis buffer (50 mm Tris pH 7.5, 1% NP-40, 0.25% Sodiumdeoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 2 µg/ml Aprotinin, 1 µg/ml pepstatin, 2 µg/ml leupeptin). Aliquots of cell lysates (70 µg protein) were resolved by 10-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto PVDF-membrane (Immobilon, Millipore, Bedford, MA) or the Hybond-P membrane (Amersham, Piscataway, NJ) and probed with the appropriate primary antibodies. Reactions were
visualized with a suitable secondary antibody conjugated with horseradish peroxidase (BioRad, Hercules, CA) by using enhanced chemiluminescence reagents (Amersham Pharmacia, Piscataway, NJ).

Drug uptake and Adduct Level assays—Cells were treated with 20µM CDDP, after which cells were washed with PBS and fresh medium was added. Cell pellets were made immediately after 1 min and 1 h of CDDP exposure (1 min, 1 h). Then, after 1 h drug exposure, cells were washed and cultured in a drug-free medium for an additional 4 h (5 h). For protein analysis, cells were digested overnight in 0.2 N NaOH at 55-60 °C. Intracellular platinum levels were determined by solubilizing the cell pellet in hyamine hydroxide and analyzed by flameless atomic absorption spectrophotometry (FAAS) using conditions previously described (detection limit = 100 pg Pt) (18,19). For platinum adduct levels, cell pellets were lysed in extraction buffer (10 mM Tris, pH 8.0, 100 mM EDTA, 20 µg/ml Rnase, 0.5% SDS) overnight at 37 °C, then treated with proteinase-K (100 µg/ml, 50 °C, 3 h) and the DNA was extracted in phenol-chloroform. The amount of platinum bound to DNA was determined by FAAS.

Immunocomplex Kinase Assays—Cells were serum-starved in 0.1% serum for 12-16 h before CDDP treatment. Whole cell extracts were prepared and treated as previously described (20). Briefly, endogenous JNK1 was immunoprecipitated from 300 µg of cell lysate with the anti-JNK1 monoclonal antibody clone 333.8 (PharMingen, San Diego, CA) and protein A-agarose beads at 4 °C for 4 h. The precipitates were washed twice in lysis buffer and twice in kinase buffer. JNK kinase activity was measured by using 2 µg of glutathione-S-transferase (GST)-c-Jun(1-79) as the substrate, and the reaction was initiated by the addition of 10 µM ATP and 10 µCi of [γ-32P]ATP (5,000 Ci/mmol) (ICN Biomedicals, Aurora, OH). After the cells were incubated at 30 °C for 30 min, the reactions were stopped with Laemmli sample buffer. The proteins were resolved by 12% SDS-PAGE and visualized by autoradiography.

Cell Proliferation Assay—Cell proliferation was assessed in 96-well plates after cells had been treated with CDDP for 1 h, washed to remove the drug and left to proliferate for the indicated time after the
addition of fresh medium. The number of surviving cells was measured by nucleic acid staining with the
CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Inc., Eugene, OR) 4-5 days after seeding. The
assay was conducted according to the manufacturer’s instructions. The samples were analyzed on a
Fluoroskan Ascent CF microplate fluorometer, made by ThermoLabSystems (Helsinki, Finland). All
experiments were carried out in quadruplicate, and the proliferation rate was expressed as the ratio of the
number of proliferating cells treated with CDDP to the number of proliferating cells not treated with
CDDP.

Flow Cytometry Analysis—To measure DNA content (apoptotic nuclei), cells were harvested, washed
with phosphate-buffered saline (PBS), fixed in 1% paraformaldehyde, stained with a solution containing 15
µg/ml propidium iodide, 0.5% Tween-20, and 0.1% RNase A, and incubated at 24 °C for 30 min. Cells
were sorted by using a Becton-Dickinson FACScan (San Jose, CA) and analyzed with CELLQuest V3.3
software (Franklin Lakes, NJ). Data were plotted on a logarithmic scale.

Detection of Fas and FasL mRNA Expression by Reverse Transcriptase-Polymerase Chain
Reaction—Total RNA was isolated from the 2008 and 2008C13 cell lines by using the RNeasy minikit
(Qiagen Inc., Valencia, CA) according to the instructions of the manufacturer. The reverse transcriptase
(RT) assay was performed from 2 µg of total RNA using Superscript II RT (Life Technologies, Grand
Island, NY) according to the manufacturer’s procedure. A reaction without RT was performed in parallel to
ensure the absence of genomic DNA contamination. Polymerase chain reaction (PCR) amplification was
carried out in a final volume of 50 µl containing 5 µl of cDNA, 5 µl of 10x PCR buffer (10 mM Tris-HCl,
pH 9, 50 mM KCl, and 0.1% Triton X-100), 0.5 µl of dNTP (10 µM), 3 µl of MgCl2 (25 mM), and 2.5 U of
AmpliTaq Gold (Perkin Elmer, Norwalk, CT). Conditions for the PCR reaction consisted of an initial
denaturation step at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at either 57 °C (for Fas)
or 61 °C (for FasL and beta-actin), and 30 s at 72 °C. After a final extension at 72 °C for 5 min, PCR
products were resolved on 1.2% agarose gels and visualized by ethidium bromide transillumination under UV light. Primer sequences were Fas forward (5'-ATT TCT GCC ACT GCA GCC CTC AGG-3') and Fas backward (5'-TCC AGT TCG CTG GGC AGA CTT CTC-3') and FasL forward (5'-ATG TTT CAG CTC TTC CAC CTA CAG A-3') and FasL backward (5'-CCA GAG AGA GCT CAG ATA CGT TGA C 3'). These sequences span nucleotides 76–706 on Fas cDNA and 365–856 on FasL cDNA and yield PCR products of 630 bp and 492 bp, respectively (21). Each reverse-transcribed mRNA product was internally controlled with beta-actin PCR by using the primers forward (5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3') and backward (5'-CTA GAA TTT GCG GTC GAC GAT GGA GGG-3'), covering the 2199–3065 region of beta-actin cDNA and giving a 867-bp PCR product (21). The FasL and Fas RT-PCR products were subsequently confirmed by direct sequencing.

Propidium Iodide and DAPI Staining—To detect apoptosis, nuclear staining was performed using 5 µg/ml of 4',6-diamidino-2-phenylindole (DAPI), and cells were analyzed with a fluorescence microscope (magnification X400 for nuclear analysis and X100 for morphologic analysis). Apoptotic cells were identified by morphology and by condensation and fragmentation of their nuclei. The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells counted, multiplied by 100. Three separate experiments were conducted and at least 300 cells were counted for each experiment.

Transfection and Immunofluorescence Staining—Expression vectors for hemaglutinin (HA) epitope tagged for wild-type c-Jun (pSRα-HA-c-Jun) and for dominant-negative c-Jun [pSRα-HA-c-Jun(A63/A73)] were a gift from M. Karin. Liposome-mediated transfection was performed by using LipofectAMINE-Plus (Life Technologies, Inc., Grand Island, NY). Briefly, the 2008 ovarian carcinoma cells were grown on chamber slides and transfected with vectors containing the HA epitope tag. After transfection, the cells were washed in PBS and fixed in methanol for 10 min at –20 ºC, after which they were air-dried, washed three times with PBS, blocked in 1.5% bovine serum albumin in PBS (PBS-BSA)
for 1 h at room temperature, and then immunostained with a monoclonal antibody to HA (dilution 1:50 in PBS-BSA) for another hour at room temperature. After 3 washes with PBS, transfected cells were visualized by incubation with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody (DAKO, Carpinteria, CA) (dilution 1:40 in PBS-BSA) for 45 min at 37 °C. To visualize the nuclei of transfected cells, we included DAPI (5 µg/ml) in the wash after the incubation with the secondary antibody. Cells were examined and photographed with an Olympus microscope (Melville, NY) equipped for epifluorescence with the appropriate filters. Transfected cells were scored blindly for apoptosis.

Detection of Cytochrome c Release—Cytosol extracts were prepared from the 2008 and 2008C13 cells essentially as previously described (22). Briefly, after cisplatin treatment and incubation for 6, 12, 18 and 24 h, the cells were collected by centrifugation. The cell pellet was washed twice with cold PBS and resuspended in ice-cold buffer A (20 mM HEPES, pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml each of leupeptin, aprotinin, and pepstatin A) containing 250 mM sucrose. The cells were homogenized with 25 strokes of a Dounce homogenizer with a type B pestle. Nuclei and intact cells were cleared by centrifugation at 1,000 x g for 10 min at 4 °C. The supernatant was centrifuged at 14,000 x g for 20 min at 4 °C to pellet the mitochondrial fraction. An aliquot of the resulting supernatant was used as the soluble cytosolic fraction. The mitochondrial pellet was washed once and then suspended in buffer A. Protein extracts (equal amounts in the mitochondrial and cytosolic fractions) were subjected to western blot analysis with a monoclonal antibody to cytochrome c.
RESULTS

*CDDP-Induced Apoptosis in Ovarian Carcinoma Cells*—CDDP-sensitive cells 2008 and CDDP-resistant 2008C13 cells were exposed to CDDP for 1 h, after which the drug was washed out to mimic *in vivo* chemotherapy. The time course for the induction of apoptosis was determined by microscopic examination of DAPI-stained cells (Fig. 1).

In the chemosensitive 2008 cells, exposure to 20 µM CDDP resulted in morphologic alterations characteristic of apoptosis, including membrane blebbing, nuclear condensation and fragmentation (Fig. 1A), and DNA laddering (data not shown). The number of apoptotic cells increased with time, and accounted for 50-70% of the total cell population by 18-24 h. The CDDP-resistant 2008C13 cells, in contrast, had a markedly different apoptotic response to this “pulsed” exposure to CDDP (Fig. 1B). Immunoblot analysis revealed cleavage of the proform of caspase-3 (32kDa) to active form of caspase-3 (17 kDa), compatible with the induction of apoptosis, from 12 to 48 h after CDDP treatment in 2008 cells but not in 2008C13 cells (Fig. 1C). PARP cleavage product also persisted from 12 to 48 h in CDDP-treated 2008 cells, as detected by immunoblot analysis, whereas in the 2008C13 cell extracts no PARP cleavage fragment were detected which correlated with the caspase-3 (Fig. 1C). An immunoblot with a β-actin antibody was used as a loading control. This finding reflected the resistance of 2008C13 cells to CDDP-induced apoptosis.

To study the mechanism behind this CDDP-resistance, we first studied drug uptake and DNA adduct formation in both the sensitive and resistant 2008 cells (Table 1). As seen, drug uptake and the DNA adduct formation were similar in the two cell lines after 1 min of CDDP treatment whereas at 1 h and 5 h after treatment, the differences between the CDDP-sensitive and CDDP-resistant cells was less than twofold, with the resistant cells showing a lower value of DNA adduct formation and drug-uptake. Since
resistance to CDDP in 2008C13 cells is greater than two fold (15,16) this has prompted us to investigate additional mechanisms of resistance.

**Differential Activation of MAPK Pathways by CDDP in Sensitive vs. Resistant Cell Lines**—Since activation of MAP kinases and phosphorylation of c-Jun have been reported after treatment with chemotherapeutic drugs in other cell types (23-27), we compared the activation of JNK between CDDP-sensitive and CDDP-resistant ovarian cancer cells after treatment with cisplatin. The activity of immunoprecipitated JNK was assayed using a GST-c-Jun fusion protein as substrate. CDDP treatment of the sensitive 2008 cells induced an increase in the ability of the JNKs to phosphorylate the GST-c-Jun substrate, beginning 1 h after treatment and persisting through the next 3-5 h (Fig. 2A); extracts from CDDP-treated resistant 2008C13 cells, on the other hand, showed only transient JNK activity after 1 h of treatment, and that activity declined rapidly over the next 3-5 h (Fig. 2A).

Next, we investigated the effect of CDDP on the phosphorylation of JNK and p38, as well as that of their respective target substrates, the c-Jun and ATF-2 transcription factors, over time. CDDP treatment of the 2008 cells, which resulted in significant apoptosis, led to sustained activation (from 1 min to 12 h after treatment) of JNK and p38, as assessed by their phosphorylation states using specific antibodies that recognize the phosphorylated (activated) forms of these enzymes (Fig. 2B). p38 MAP kinase activation occurred over the same period as that of JNK. Although phosphorylation was detected very early (at 1 min), maximal phosphorylation of both kinases became apparent at about 1 h and was sustained over the following 12-h period (Fig. 2B, left panel). Consequently, phosphorylation of c-Jun at S73 and ATF-2 at T71 occurred over the same extended period in 2008 cells (Fig. 2B, left panel). This sustained phosphorylation did not result from increased expression of either JNK or p38 as protein levels were unaltered relative to untreated cells (Fig. 2B, left panel). In contrast, the CDDP-resistant variant 2008C13 showed only transient (1-3 h after treatment) JNK and p38 phosphorylation, a pattern that was reproduced
in phosphorylation of the c-Jun and ATF-2 target substrates (Fig. 2B, right panel). Similar results were found with regard to the differences in duration of JNK activation and c-Jun phosphorylation in studies of another ovarian carcinoma cell with CDDP-sensitive and -resistant variants (A2780 and A2780CP) (data not shown).

Next, we assessed whether cisplatin might activate the ERK pathway in ovarian cells, and we found that CDDP induced transient ERK phosphorylation in both the 2008 and the 2008C13 cell lines. Phosphorylation of ERK was detected at a 1 h time point in the 2008 cells, but an early, somewhat persistent phosphorylation (1 min to 1 h) was observed in the 2008C13 cells (Fig. 2B). This ERK phosphorylation pattern was not sustained and was the opposite of the phosphorylation pattern seen for the JNK and p38 MAPKs. In the resistant cells, ERK phosphorylation also seemed to be biphasic with a second increase occurring at 12-16 h. These data agree with previous findings that CDDP induced apoptosis correlates with an increase in JNK and c-Jun phosphorylation in other cell types (23,26,27). Our findings also suggest that the difference in duration of the activation of the JNK and p38 MAP kinase pathways in CDDP-sensitive and -resistant cells could contribute to directing the outcome to survival or apoptosis.

Absence of c-Jun or Inhibition of JNK Pathways Confers Resistance to Apoptosis and Increases Cell Survival After Treatment with CDDP—The delay between JNK (or p38) activation and the onset of apoptosis, together with the requirement for prolonged JNK (or p38) phosphorylation, suggests that expression of new genes may be required to activate apoptosis. Because the c-Jun transcription factor is an important and specific target for JNK (10) and is possibly involved in the apoptosis triggered by DNA-damaging agents (26,27), we hypothesized that prolonged activation of the JNK and p38 pathways induced by CDDP is required to induce apoptosis in a manner dependent on c-Jun transcription. Conversely, we
also hypothesized that a transient activation or immediate inactivation of these kinase pathways is too brief to transactivate AP-1-responsive genes and thus would lead to resistance to CDDP-induced apoptosis.

To further investigate the involvement of c-Jun activation in CDDP-induced apoptosis, we compared immortalized 3T3 fibroblast cell lines that have a targeted disruption of the c-jun gene (28) with their parental 3T3 cells that express a wild-type (wt) c-jun. We first analyzed whether the absence of the c-jun gene affected cell survival after CDDP exposure. As shown in Fig. 3A, as indicated by DAPI-staining, the CDDP-treated c-jun<sup>−/−</sup> cells were significantly more resistant to apoptosis than were parental cells. About 80% of the c-jun<sup>−/−</sup> 3T3 cells survived CDDP treatment, but only 25% of the wt c-jun 3T3 cells were still viable after 4–5 days, suggesting that activation of a downstream set of target genes through c-jun leads to CDDP-induced apoptosis. To determine the possible role of the specific members of the MAP kinase family in mediating this process, we tested the parental 3T3 cells and c-jun<sup>−/−</sup> 3T3 cells with the pyridinylimidazole compound SB202190, a strong inhibitor of JNK and the p38/HOG kinase (29-31). Survival measurements revealed that pretreatment with SB202190 led to a significant increase in cell survival in both the absence and the presence of c-jun (Fig. 3A). Inhibition of JNK and p38 decreased the effect of CDDP cisplatin in wild-type c-jun 3T3 cells (72% survived with SB202190 pretreatment versus 26% without SB202190). Results of an immune complex kinase assay, which showed a decrease in phosphorylation of GST-c-Jun by immunoprecipitated JNK (Fig. 3B), confirmed that SB202190 treatment inhibited CDDP-induced c-Jun N-terminal phosphorylation and that this inhibition of c-Jun phosphorylation correlated with the inhibition of apoptosis (Fig. 3A).

These data confirm that c-Jun and its activation by JNK were required for efficient induction of apoptosis by the alkylating agent CDDP. Blockade of JNK activation, whether by a small drug inhibitor or by a lack of c-jun, protected cells from cisplatin-induced apoptosis.
A JNK and p38 Inhibitor Prevents CDDP-induced Apoptosis—To further investigate whether the JNK pathway was required for CDDP-induced apoptosis, we pretreated 2008 cells with SB202190 under conditions in which both JNK and p38 activation were inhibited (data not shown) before CDDP exposure. Incubation with SB202190 resulted in a marked reduction in cell death (Fig. 4A). Cells treated with CDDP alone, however, displayed the typical features of apoptosis: condensation of the nuclei (Fig. 4A, left panel), shrinkage of the cytoplasm, and membrane blebbing (as seen by phase contrast microscopy; data not shown). Interestingly, pretreatment of the cells with SB202190 markedly suppressed the morphologic changes induced by CDDP (Fig. 4A, left panel). To confirm these findings with an independent assay, we measured apoptosis by propidium iodide staining and flow cytometry. At 24 h after treatment with CDDP, 51% of the 2008 cells showed a hypodiploid (sub-G₁) DNA content, reflecting apoptosis (Fig. 4B). However, incubation with SB202190 before CDDP treatment reduced the extent of cell death considerably, from 51% to 9% (Fig. 4B) and inhibited CDDP-induced cell death in a dose-dependent manner (data not shown).

To determine whether phosphorylation of the c-Jun transactivation domain was necessary for the induction of apoptosis, we transfected 2008 cells with either wild-type (wt) c-Jun or an HA-c-Jun(A63/73) in which the serines at 63 and 73 that are normally phosphorylated by JNK were replaced by alanine, resulting in an inactive protein (20). Transient expression of the HA-c-Jun(A63/73) was associated with a marked decrease in CDDP-induced apoptosis compared with the level seen in cells transfected with wt c-Jun, (Fig. 4C). This protective effect was restricted to the c-Jun(A63/73)-expressing cells, whereas in the surrounding cells that did not express this protein, the induction of apoptosis was not inhibited. As seen in Fig. 4C, about 30% of 2008 cells expressing wt c-Jun were undergoing apoptosis when compared to only 7%-8% of 2008 cells expressing the HA-c-Jun(A63/73). These data confirm the hypothesis that either c-Jun phosphorylation or JNK activation is required for CDDP-induced apoptosis and that inhibition of this pathway could exert a protective effect.
Expression of FasL in Response to CDDP is Impaired in 2008C13 Resistant Variants But Not in CDDP-Sensitive Ovarian Carcinoma Cells—Because CDDP’s ability to induce apoptosis seems to depend on its ability to activate JNK- and c-Jun-dependent transcriptional events, we next sought to identify JNK and c-Jun targets that might mediate CDDP-induced apoptosis.

To determine whether the Fas ligand gene is a target of CDDP-dependent c-Jun activation, we examined whether the exposure of ovarian carcinoma cells to CDDP affected the expression of FasL. Treatment of the CDDP-sensitive 2008 cells with CDDP led to an up-regulation of FasL (Fig. 5A) that began at 6 h and peaked at 18-24 h, a time span that corresponds to the kinetics of apoptotic cell death (Fig. 1B). In contrast, no FasL mRNA was detected in the CDDP resistant 2008C13 cells (Fig. 5A, lanes 5-8), even at higher concentrations of CDDP (40 µM) (Fig. 5A, lanes 14-18). Fas receptor mRNA expression levels did not change after CDDP treatment and were comparable in both cell lines (Fig. 5A). These data identify the FasL gene as a c-jun-regulated target gene for CDDP and suggest that lack of FasL induction may contribute to the CDDP-induced apoptosis defect in the resistant 2008C13 cells. To explore this possibility, we examined whether blocking Fas/FasL interaction after CDDP treatment would protect cells from undergoing apoptosis. We used the NOK-2 antibody, which recognizes and neutralizes both membrane-bound and soluble forms of human FasL, thereby preventing the interaction of either form with Fas. Incubation of the 2008 cells with this neutralizing anti-FasL IgG antibody after CDDP treatment protected the cells from CDDP-induced apoptosis by 30% to 40% at 24 h and 48 h, respectively, compared with the level of apoptosis in cells that had been preincubated with an isotype IgG control (Fig. 5B).

Because interfering with the Fas/FasL system is known to reduce sensitivity to drug-mediated apoptosis in some cell systems (32-34), we next determined whether Fas/FasL function influenced apoptosis in the resistant 2008C13 cells by treating the cells with a Fas antibody that binds to the Fas antigen, a process that mimics the role of FasL in the induction of apoptosis. The extent of Fas-mediated
apoptosis was similar in the sensitive and the resistant cells at 24 and 48 h (Fig. 5C). These results suggest that the Fas receptor that was expressed on the surface of both resistant and sensitive 2008 cells was functional and could trigger apoptosis. Taken together, these data show that both FasL up-regulation and Fas/FasL interactions were important for the induction of apoptosis in ovarian carcinoma cells after their exposure to CDDP.

To determine whether the JNK and p38 MAK kinase pathway in ovarian cancer cells was involved in the FasL-mediated apoptosis induced by CDDP, we tested whether inhibiting JNK and p38 with the small drug inhibitor SB202190 would block FasL expression. We found that pretreatment of the 2008 cells with SB202190 significantly inhibited FasL mRNA induction in response to CDDP (Fig. 5D) but had no significant effect on Fas-mediated apoptosis (Fig. 5E). The effect of this inhibition on FasL transcriptional activity might have been due to the inhibition of AP-1 activity, because the SB202190 completely inhibited JNK activation and therefore c-Jun phosphorylation (29-31). Taken together, these findings strongly suggest that FasL induction was required for CDDP-induced ovarian carcinoma apoptosis and that this process depended on JNK and the phosphorylation of c-Jun at serines 63 and 73. However, incubation of cells with SB202190 did not prevent Fas/FasL from triggering apoptosis (Fig. 5E), suggesting that the JNK pathway might not be downstream or might not be required for Fas-mediated apoptosis.

_Caspase Inhibition Does Not Block CDDP-Induced FasL Expression—_Initiation of apoptotic cell death and caspase activation in response to various stimuli, including CDDP, requires the release of cytochrome _c_ from the mitochondrial intermembrane space into the cytosol (35). In the cytoplasm, cytochrome _c_ promotes the assembly of a protein complex called the apoptosome, which includes caspase-9 bound to the CED-4 homologue Apaf-1 (36,37). Upon activation, caspase-9 instigates a proteolytic cascade involving multiple caspases, a process culminating in the cleavage of numerous substrate proteins and, ultimately, cell death (38). Activation of caspase-3 and cleavage of PARP cleavage was seen during
12h-48h after CDDP treatment, in the CDDP-sensitive cells undergoing apoptosis (Fig 1C). Caspases may function both upstream and downstream of Fas/FasL in the apoptosis signaling pathways. To examine further the effect of caspase activation on CDDP-induced apoptosis in the 2008 cells, we used the pan-caspase inhibitor zVAD-fmk to determine whether activation of FasL was linked to the caspase activation leading to apoptosis after CDDP treatment. We treated the 2008 cells with CDDP with or without zVAD-fmk (50 µM) and examined them at 18 and 24 h after the treatment. As shown in Fig. 6, zVAD-fmk substantially attenuated CDDP-induced PARP cleavage and apoptosis, which was determined by visualization of DAPI-stained cells (data not shown); however, zVAD-fmk had no effect on CDDP-induced FasL induction (Fig. 6A-C). These findings indicated that in ovarian carcinoma cells, CDDP induced FasL up-regulation is upstream of caspase activation.

Recent findings have indicated that activation of the JNK pathway influences cytochrome c release and that apoptotic stimuli fail to release cytochrome c in JNK-null cells (39). To determine whether cytochrome c is released in response to treatment with CDDP and participates in the JNK pathway, 2008 cells were treated with CDDP, after which cytosolic fractions and mitochondrial proteins were extracted and tested for cytochrome c release by immunoblotting. Cytochrome c level decreased in the mitochondria and increased in the cytoplasm of the 2008 cells, but not the 2008C13 cells, at 18-24 h after CDDP treatment (Fig. 6D). This observation agreed with our other findings on the extent and time course of PARP cleavage and apoptosis in these cells (Figs. 1 and 6A and 6B). Whereas JNK activation in the 2008 cells was detected earlier (1 min to 5 h) after CDDP treatment (see Fig. 2), cytochrome c release was markedly delayed, starting only after 12 h (Fig. 6D). These findings are compatible with JNK acting upstream of the cytochrome c release. Notably, cytochrome c release was abrogated in the resistant 2008C13 cells, even at 24 h after the CDDP treatment (Fig. 6D). Thus, it seems that the cells selected for resistance to CDDP have defects in the release of cytochrome c and in the resulting activation of downstream caspases.
Constitutive Activation of JNK/p38 MAP Kinases through MKK7/3 Sensitizes Chemoresistant 2008C13 Cells to CDDP-Induced Apoptosis by Inducing FasL Expression—That the chemoresistant subclone 2008C13 showed only a transient phosphorylation of JNK and p38 in response to CDDP, compared with the sustained JNK and p38 phosphorylation seen in the lysates of the chemosensitive 2008 cell line (Figs. 1 and 2) suggest that the duration of JNK and p38 phosphorylation seems to play an important role in the regulation of apoptosis in ovarian cancer cells, since the Moreover, inhibition of the JNK and p38 kinases by SB202190 prevented CDDP-induced FasL expression (Figs. 4 and 5D). Possibly, the failure of the JNK and p38 kinases to be activated owing to their inactivation by specific MAP kinase phosphatases that are induced in response to genotoxic stress (40) could underlie chemoresistance. We therefore examined whether selective reactivation of endogenous JNK and p38 could induce or sensitize the resistant subclone 2008C13 to apoptosis. To test this hypothesis, we used recombinant adenoviruses encoding constitutively active MKK7 (MKK7D) to selectively induce JNK activity (41,42) or MKK3 (MKK3bE) to selectively induce p38 activity (43). The efficiency of the adenovirus gene delivery system in these ovarian cancer cells reached 90%, and the results showed phosphorylation of JNK and p38 after infection with Ad-MKK7D and Ad-MKK3bE, respectively, as compared with infection with Ad-GFP (Fig. 7A). These data indicate that activated MKK7D and MKK3bE functioned as JNK- and p38-specific activators in ovarian tumor cells in the same fashion as previously demonstrated in other cell types (43). Moreover, FasL was upregulated when MKK7D and MKK3bE were transduced, and FasL upregulation correlated with the rate of apoptosis (Fig. 7B, C). Control virus expressing GFP had no effect (Fig. 7B, C). Taken together, these results suggest that activation of the JNK and p38 pathways through MKK7D and MKK3bE respectively, are sufficient to induce expression of FasL and promote apoptosis in resistant ovarian carcinoma cells. These data are consistent with our previous observations that failure to activate JNK and p38 led to a survival/chemoresistant phenotype and that persistent activation of JNK and p38
promoted apoptosis through FasL gene expression in the CDDP-sensitive 2008 ovarian carcinoma cell line (Figs. 2, 4, and 5).

**DISCUSSION**

Primary and secondary resistance to chemotherapy is a central problem in cancer treatment (1,4,9). Resistance to chemotherapy may result from a failure of the apoptosis pathways that are activated in response to drug treatment. Recent evidence indicates that the MAPK family protein kinases JNK and p38 are important mediators of the apoptosis induced by stressful stimuli (25,31,39,44,45). The JNKs and p38 MAPKs are collectively termed stress-activated protein kinases because they are activated by a variety of stress-related stimuli [for review see (10,11)]. The stress kinases are also activated by chemotherapy drugs, including paclitaxel, doxorubicin, vinblastine, and etoposide (46), and by certain DNA-damaging agents, such as 1-D-arabinofuranosyletosine, CDDP, and mitomycin C (24,46,47).

JNK activity parallels c-Jun phosphorylation in intact cells, suggesting that this protein kinase plays an important role in regulating c-Jun transcriptional activity. JNK activation results in the phosphorylation of transcription factors such as c-Jun and ATF-2, which then bind to AP-1 binding sites in the promoters of multiple target genes. JNK may contribute to death receptor transcription-dependent apoptotic signaling via c-Jun/AP-1 leading to transcriptional activation of FasL. JNK was initially thought to be a mediator of apoptosis in neuronal cells (44) and phosphorylation of c-Jun was shown to be essential for neuronal cell death induced by withdrawal of survival-signals (31,48-50).

The results described in this study map the early signaling events by which activation of JNK and p38 after CDDP treatment can lead to apoptosis in ovarian cancer cells. Previous studies have described the link between induction of JNK activation and apoptosis in response to cisplatin treatment in ovarian cells and other cell types (25-27). Those studies also suggested that the transcriptional activity of the c-Jun protein, which is increased by phosphorylation of c-Jun at serines 63 and 73 by JNK, is closely associated
with apoptosis. However, none of those studies indicated a potential mechanism by which JNK activation, c-Jun phosphorylation, or both could trigger apoptosis.

Our findings demonstrate that prolonged phosphorylation (1 min to 12 h) of JNK and p38 MAP kinase, accompanied by c-Jun/ATF-2 phosphorylation, is an important step in the apoptosis-signaling cascade induced by CDDP. More importantly, this sustained JNK activation and c-Jun phosphorylation paralleled the phosphorylation of p38 and ATF-2 (Fig. 2). These effects preceded and triggered up-regulation of FasL, which in turn contributed to the apoptotic response (Figs, 1, 2, 4, and 5). Thus, the duration of JNK and p38 pathway signaling is a critical factor in determining cell survival or apoptosis; transient activation was insufficient to induce death in CDDP-resistant cells, and prolonged JNK and p38 activation triggered cell death in CDDP-sensitive cells. Our findings indicate that resistance to CDDP in ovarian carcinoma cells is due in part to lack of prolonged activation of stress kinases and phosphorylation of c-Jun and ATF-2, a c-Jun dimerization partner. The transient activation observed in resistant cells seems to be insufficient to induce gene expression of a major initiator of apoptosis, FasL (Fig. 5). This differential response to CDDP between 2008 and 2008C13 cells may be due to differences in cellular uptake and induced DNA damage (Table 1) is unlikely since expression of FasL was uninduced in 2008C13 cells even after a two-fold increase in the drug concentration (Fig. 5A).

We also showed that inhibition of c-Jun activity, either by using a mutant defective in the JNK phosphoacceptor sites [c-Jun(A63/73)] (Fig. 4C) or by inhibiting JNK activation with the small drug inhibitor SB202190 (Fig. 4A, B) could block CDDP-induced apoptosis. Most importantly, this blockade correlated with c-Jun’s inability to activate FasL gene expression (Fig 5D). In line with this evidence, others have reported that activation of JNK is required for an apoptotic response to alkylating agents (26,27,39,51). Several studies have suggested that c-Jun is involved in genotoxin-induced apoptosis (52). One study demonstrated that a dominant-negative c-Jun mutant reduced apoptosis in human monoblastic leukemia cells after exposure to various DNA-damaging agents (53). Both c-jun<sup>−/−</sup> fibroblasts and Jnk1<sup>−/−</sup>
Jnk2−/− double-knockout murine embryonic fibroblasts were found to be resistant to apoptosis induced by UV irradiation, anisomycin, and alkylating agents (39,51,52,54), all of which may be mediated by the induction of FasL (52). Our data are also consistent with a previous demonstration that long-lasting activation of JNK and p38 kinase after withdrawal of survival factors or induction of MEKK1Δ resulted in enhanced c-Jun phosphorylation and in induction of FasL, leading to neuronal cell death (31,50). Further, the direct inhibition of JNK or c-Jun can block neuronal apoptosis induced by survival-factor withdrawal (44,55,56). Most importantly, mice harboring a mutant allele of c-jun with serines 63 and 73 mutated to alanines are resistant to neuronal apoptosis induced by kainate (48). However, the normal physiological function of c-Jun or JNK, even in the context of a stress response, does not necessarily include induction of apoptosis (10,11).

Tumor cells can inactivate pro-apoptotic cytokines such as Fas/FasL in a way that confers resistance to chemotherapy (8). Furthermore, it is most likely that upstream MAK kinases are involved in this pathway, since selective reactivation of JNK or p38 kinase by MKK7 or MKK3 induced apoptosis in the chemoresistant cells through transcriptional up-regulation of FasL expression (Fig. 7). Interestingly, activation of both MKK6 and p38 is required for gamma-irradiation-induced G2 arrest, and the expression of dominant-negative alleles of MKK6 or p38 allows cells to escape the DNA damage–induced G2 delay (57). Zanke et al. reported, for instance, that cell lines defective in JNK activation were resistant to the lethal effects of CDDP (25). Moreover, either the expression of a dominant-negative SEK1/MKK4 mutant that blocks JNK activation or the transient expression of a dominant-negative JNK1 mutant was sufficient to confer resistance to apoptosis induced by several different stressful stimuli, including heat shock, UV irradiation, and a 2-h exposure to CDDP (25). These findings support the concept that the JNK pathway plays an important role in c-Jun-induced apoptosis. They also suggest that activation of the JNK pathway by diverse cell stress agents play a critical part in mediating the toxicity of these treatments, including cell
death. JNK activation in this context could broadly influence the cellular response of tumor cells to cytotoxic therapies.

In the present study, we demonstrated that expression of the AP-1 target gene, FasL, whose product can promote apoptosis, was highly induced by CDDP in 2008 cells and that this induction correlated with a persistent activation of JNK and p38 and c-Jun phosphorylation. On the other hand, FasL expression was impaired in the CDDP-resistant variant 2008C13, even at a higher dose of CDDP (40 µM), and this impairment correlated with a transient JNK phosphorylation (Figs. 2 and 5). The addition of a purified anti-Fas antibody resulted in significant and comparable apoptosis, indicating that Fas-induced cell death pathway was functional in both cell types (Fig. 5C). Fas and FasL are a cognate receptor-ligand pair and play central roles in regulating programmed cell death. Interaction with FasL induces trimerization of the Fas receptor, leading to the recruitment of adaptor molecules such as the Fas-associated death-domain protein (FADD), which directly binds and activates caspase-8, resulting in the induction of apoptosis. Activation of the Fas/FasL system is known to occur in a range of tumor cell lines after exposure to various types of anticancer drugs (32,34). However, no clear explanation exists as to why some activators of AP-1 lead to FasL induction while others do not. Kasibhatla et al. initially reported that death ligands are subject to transcriptional regulation and that the FasL promoter therefore was directly activated by c-Jun through an AP-1 binding site in transient transfection experiments (33). Other studies have shown that withdrawal of survival factor or activation of the JNK pathway leads to an induction of apoptosis that is preceded by up-regulation of an immediate downstream target, the Fas ligand (FasL), in cerebellar granule neurons and PC12 cells (31,33,58). In addition, inhibition of the interaction of Fas ligand with its receptor Fas leads to a reduction in apoptosis in response to genotoxic stress and growth factor withdrawal (31,33,52,58). Consistent with the idea that the Fas ligand is a target in the JNK-c-Jun signaling pathway that induces apoptosis is the presence of AP-1 binding sites in the human FasL promoter region, which presumably contribute to the dependence of FasL-Fas interactions on c-Jun phosphorylation (31,33,58,59). Indeed,
several reports have identified AP-1 sites in the FasL promoter that are recognized by Jun-Fos or c-Jun-ATF-2 heterodimers. The presence of these sites is required for optimal responsiveness to such cellular stresses as exposure to UV gamma-irradiation and to alkylating agents (33,52,59,60).

In ovarian tumors, FasL may be a pro-apoptotic target of JNK/AP-1 signaling, since inhibition of JNK and p38 with SB202190 led to inhibition of the induction of FasL mRNA (Fig. 5D), and, conversely, reactivation of FasL induction by activated MKK7 or MKK3 (which induced persistent activation of JNK or p38, respectively) triggered cell death through Fas-L expression (Fig. 7). Interestingly, sustained suppression of Fas/FasL has been reported in CDDP-resistant cells, suggesting that the inability of these cells to up-regulate these receptors and ligands may be an important determinant of the ability of the cells to undergo apoptosis in response to chemotherapeutic agents (8). Clearly, c-Jun is required for FasL up-regulation since c-Jun-deficient fibroblasts, in contrast to wild-type cells, exhibited a defect in CDDP-induced apoptosis, and since the inhibition of JNK by SB202190 significantly prevented CDDP-induced cell death in wild-type cells (Fig. 3A). In support of our findings is the fact that c-Jun-dependent FasL induction has been demonstrated in several systems in response to DNA-damaging agents, including the topoisomerase II inhibitors, UV irradiation, and the alkylating agent methyl methanesulfonate (21,33,52). Kolbus et al. also showed that the resistance of c-jun−/− fibroblasts to apoptosis was accompanied by impaired expression of the FasL providing evidence that c-Jun-dependent expression of FasL represents a rate-limiting step in the apoptosis induced by methyl methanesulfonate (52). Thus, lack of c-Jun activity increases survival to genotoxic stresses (52,54), and reintroduction of the c-jun gene into c-jun−/− fibroblast cells sensitizes cells to UV-induced apoptosis (54). The loss-of-function approach in fibroblasts allowed the identification and dissection of c-Jun-dependent and -independent processes upstream and downstream of Fas activation. Once activated, Fas-induced death signaling is not affected by the loss of c-Jun nor JNK activation, demonstrating that only the initiation and not the execution of stress-induced apoptosis depends on c-Jun (Fig. 5E) (52). These data strongly suggest that one mechanism underlying chemoresistance might
be the inability to sustain activation of stress kinases and therefore to enable up-regulation of the
downstream target death gene, FasL.

Downstream consequences of Fas/FasL interaction are complex and depend in part on the cell type
being studied. Some studies implicate JNK in apoptosis and others describe a lack of correlation between
JNK and cell death or even interference of JNK activation with apoptosis [for reviews, see (10,11)]. The
present study demonstrated that JNK and p38 activation is not due to a downstream FasL signaling event,
since JNK and p38 activation occurred during the first hour (i.e., before FasL was expressed 6-18 h later)
(Figs. 2 and 5). Moreover, the caspase inhibitor zVAD-fmk did not prevent CDDP-induced FasL (Fig. 6) or
JNK and p38 activation (data not shown), indicating that FasL expression is an event downstream of the
JNK/p38 pathway. Also in support of our findings are reports that treatment with recombinant FasL in
wild-type 3T3 cells and c-jun−/− cells did not result in any detectable JNK activity [(52) and data not
shown]. However, in the studies of Kolbus et al., both cell lines responded to methyl methanesulfonate by
showing activation of kinase activities within the first hour, and no additional increase in JNK activity was
observed at later times, when FasL induction reached maximal levels and apoptosis became detectable.
Clearly, neither c-Jun nor JNK are required for the expression and activity of cellular components located
downstream of Fas, because c-jun−/− cells and jnk1−/− jnk2−/− mouse embryonic fibroblasts were sensitive to
FasL-induced apoptosis (39,52).

The molecular pathways triggered by anticancer drugs that lead to the activation of stress pathways
are not well understood. Exactly how cisplatin triggers stress kinase pathways is not yet known, nor are the
sequential events between CDDP-induced oxidative stress, DNA damage, JNK/p38 activation, and
apoptosis. We have also reported that cisplatin-resistance in the ovarian carcinoma is associated with a
defect in apoptosis through X-chromosome-linked inhibitor of apoptosis (XIAP) regulation (61). Based on
our data and literature reports, several hypotheses can be proposed. It is clear that powerful pro-oxidants
that cause generation of reactive oxygen species and free radicals are generated in response to nonredox-
active initiators of apoptosis such as cisplatin. In addition, studies have shown that pretreatment of cells with the antioxidants glutathione or N-acetyl-cysteine effectively blocks CDDP-induced apoptosis and CDDP-induced activation of JNK and p38 (57,62,63). Finally, another possibility in the control of stress kinase activities in response to CDDP may relate to interference with phosphatases (64). MAP kinase phosphatases play an important role in selectively regulating the duration of JNK and p38 phosphorylation and dephosphorylation (65) and are activated by various stresses that activate JNK. All known phosphotyrosine and threonine phosphatases, including the dual-specificity phosphatases, contain an essential catalytic cysteinyl residue (66) that is sensitive to thio-(SH)-reactive agents such as CDDP. Therefore, oxidative stress generated by CDDP not only may deplete reduced glutathione and other antioxidant molecules but also may cause the oxidation of the sulhydryl groups on these phosphatases, leading to their inactivation (51,67). Our study shows that the sensitization of carcinoma cells to genotoxic stress is largely due to potentiation of the JNK and p38 pathways. Indeed, the CDDP-resistant 2008C13 cells exhibited a defect in the activation of JNK that may contribute to the resistance of advanced tumors to cancer therapy. This suppression of stress kinase activation supports the possible role of an alteration of phosphatase activities in CDDP-resistant cells. Thus, the specific induction of MAP kinase phosphatase by these agents seems to be responsible for protecting cells from apoptosis by preventing prolonged activation of JNK/p38 kinases (51,67). The duration of JNK/p38 activation could thus be regulated by MAP kinase phosphatases through a feedback mechanism. Whether CDDP inactivates JNK in resistant cell lines via stimulation of MAP kinase phosphatases is under investigation by our group.

In conclusion, we have demonstrated that an early key determinant of CDDP-induced apoptosis is the duration of JNK and p38 phosphorylation. Prolonged JNK and p38 activation results in phosphorylation of the target AP-1 transcription factors c-Jun and ATF-2, thus promoting the expression of FasL and binding of FasL to the Fas receptor, which leads to cell death (Fig. 8). Since in CDDP-sensitive cells stress kinase pathways remain potentiated, the cells remain sensitive to stress signals such as
genotoxic agents. These findings raise the possibility that defects in this cascade may contribute to a failure of chemotherapy-induced apoptosis. Therefore, modulation of apoptotic pathways through the MAP kinase signaling cascade may become a therapeutic goal for the prevention and treatment of cancer.

ACKNOWLEDGMENTS

We are grateful to S. B. Howell, S. G. Chaney, and Z. H. Siddik for kindly providing the 2008 and 2008C13 cells and for sharing data; to M. Karin and E. F. Wagner for the gift of c-jun+/+ and c-jun−/− fibroblasts and plasmids; and to N. Hollbrook and O. Potapova for sharing their unpublished data. A.M. is a recipient of fellowships from the Association pour la Recherche sur le Cancer and Fondation pour la Recherche Medicale. This study was supported by funding from The University of Texas M. D. Anderson Cancer Center and grants from the National Institutes of Health (P30CA16672-24, 5P50CA83639, and Core Grant CA16672) and the Ovarian Cancer Research Program of the U.S. Department of Defense to F.X.C.

FOOTNOTES

1The abbreviations used are: CDDP, cisplatin; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; ERK, extracellular signal-regulated kinase; FasL, Fas ligand; ATF-2, activating transcription factor 2; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PARP, poly (ADP-ribose) polymerase; DAPI, 4,6-diamidino-2-phenylindole; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; GST, glutathione S-transferase; PBS, phosphate-buffered saline; RT, reverse transcriptase; PCR, polymerase chain reaction; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-(O-methyl)-fluoromethyl ketone; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.
TABLE I
CDDP uptake and CDDP-DNA adduct formation in 2008 and 2008C13 cells

| TIME* | 1 min    | 1 h      | 5 h      |
|-------|----------|----------|----------|
| UPTAKE (ng Pt/mg prot.) |          |          |          |
| 2008  | 11.14 ± 1.97 | 81.88 ± 1.61 | 36.81 ± 0.94 |
| 2008C13 | 15.84 ± 0.98 | 44.59 ± 2.31 | 26.90 ± 0.03 |
| DNA ADDUCTS (ng Pt/mg DNA) |          |          |          |
| 2008  | 6.89 ± 0.64  | 147.03 ± 3.41 | 74.22 ± 1.03 |
| 2008C13 | 6.06 ± 0.13  | 69.29 ± 2.76  | 55.12 ± 1.52 |

(*) after initiation of CDDP treatment
FIGURE LEGENDS

Figure 1. CDDP induces massive apoptosis in chemosensitive 2008 cells but only moderate apoptosis in chemoresistant 2008C13 ovarian carcinoma cells. A, Representative micrographs illustrating morphologic evidence of apoptosis as assessed by nuclear staining 24 h after CDDP treatment. 2008 and 2008C13 cells were treated with 20 µM of CDDP for 1 h, then cultured in a CDDP-free fresh medium for the remaining time, after which cells were fixed and nuclear condensation was analyzed with DAPI. Typical morphologic changes associated with apoptosis were visualized by fluorescence microscopy (X400). B, CDDP induced apoptosis in ovarian carcinoma cells in a time-dependent manner. 2008 and 2008C13 cells were treated for 1 h with 20 µM CDDP and collected at the indicated times (right panel). Cells were then subjected to DAPI staining and fluorescence microscopy counting as in (A). The results shown are the average of five experiments. C, Immunoblot analysis illustrating differential effect of CDDP on activation of caspase-3 and cleavage of the caspase substrate PARP in the two cell lines. Cells were treated as in (A) and then harvested, and their protein extracts were analyzed by SDS-PAGE using an anti-caspase-3, anti-PARP antibodies and anti-actin antibody as a loading control.

Figure 2. Activation of MAPK signal-transduction pathway in response to CDDP in the ovarian cancer cell lines 2008 and 2008C13. A, Effect of CDDP on JNK activity. Cells were kept in 0.1% serum for 12-16 h and were either exposed to 20 µM CDDP for 1 h or left untreated (NT). At 1, 3, and 5 h after completion of CDDP treatment, cells were collected, lysed, and subjected to JNK1 immune-complex kinase assays using the GST-cJun(1-79) fusion protein as a substrate. Equal number of cells were harvested at the indicated times. B. Time course of CDDP-induced p38, ATF-2, JNK, c-Jun, and Erk phosphorylation. CDDP-sensitive (2008) and -resistant (2008C13) human ovarian carcinoma cells were...
exposed for 1 h to 20 µM CDDP or left untreated (0). At the indicated times after completion of CDDP treatment, whole-cell extracts were prepared, and protein extracts were resolved by SDS-PAGE and immunoblotted with antiphospho-p38 and antiphospho-ATF-2, antiphospho-JNK or antiphospho-c-Jun, and antiphospho-ERK1/2, which recognize the activated forms of p38, JNK and ERK, respectively. The total amount of p38, ATF-2, JNK1, and c-Jun proteins was assessed using antibodies that recognized these proteins independent of their phosphorylation status.

Figure 3. CDDP-induced apoptosis depends on c-Jun. A, Wild-type c-Jun or c-Jun−/− 3T3 cells were left in 0.25% serum for 12 h and treated with the JNK-activation inhibitor SB202190 at 30 µM for 1 h. The cells were then treated with 100 µM CDDP for 1 h, cells were washed four times to remove the drug and fresh medium was added containing 0.25% serum. Cell viability was measured 4 days later by using a fluorescence-based nucleic acid method (the CyQUANT cell proliferation assay kit). B, SB202190 inhibited CDDP-induced JNK activity in 3T3 cells. Wild-type c-Jun or c-Jun−/− 3T3 were treated for 1 h with or without SB202190 as in (A) before exposure to CDDP for 1 h. Cell lysates were prepared 1 h after CDDP treatment, and JNK activity was analyzed in an immune-complex kinase assay using GST-cJun(1-79) as a substrate.

Figure 4. Effect of the p38/JNK MAPK inhibitor SB202190 on CDDP-induced apoptosis in CDDP-sensitive cells. A, Morphologic changes in chemosensitive 2008 cells at 24 h after a 1-h treatment with CDDP either followed by or preceded by treatment with SB202190. Control cells were untreated. Apoptosis was visualized after DAPI staining by fluorescence microscopy X400 (left panel). Quantitative analysis is shown at right. B, Flow cytometry of propidium iodine-stained untreated 2008 cells and cells treated with CDDP and SB202190 as in (A). Cells were stained with propidium iodine, and the number of apoptotic cells was counted with a FACScalibur flow cytometer. The percentages of apoptotic cells are indicated as the proportion of cells that contained sub-G1 DNA. Representative results from two
independent experiments are shown. C, A phosphorylation-defective c-Jun mutant inhibited CDDP-induced cell death in 2008 cells. Cells cultured on glass coverslips were transfected with HA-tagged c-Jun wt or with c-Jun(A63/73) expression vectors. After 24 h, the cells were washed with PBS and incubated for 1 h with 20 µM CDDP. The cells were subsequently fixed and permeabilized, and expression of FITC-labeled HA-c-Jun proteins was detected by indirect immunofluorescence using a monoclonal HA antibody. Nuclear morphology was visualized by staining with DAPI. The white arrows indicate cells transfected with either wt HA-c-Jun (cells have apoptotic morphology) or HA-c-Jun(A63/73) (cells have nonapoptotic morphology). The gray arrows indicate surrounding cells with apoptotic morphology. Lower panel represents a quantification of Fig 4C, where at least 300 cells were counted for each experiment.

**Figure 5.** CDDP leads to up-regulation of FasL in chemosensitive 2008 cells but not in chemoresistant 2008C13 cells. A, FasL mRNA was induced in 2008 cells and abrogated in 2008C13 cells after stimulation with CDDP. Cells were treated with CDDP, either at 20 µM (left panel) or 40 µM (right panel). Total RNA was purified at the indicated times, and the expression of FasL, FasR, and beta-actin mRNAs was examined by RT-PCR using specific primers. B, An anti-FasL neutralizing antibody (NOK-2) abrogated CDDP-induced apoptosis. Ovarian carcinoma 2008 cells were treated with CDDP for 1 h, and then the cells were cultured in the presence of either NOK-2 antibodies (50 µg/ml) or an isotype-matched control immunoglobulin (Ig) (50 µg/ml). At 0, 6, 12, 24, and 48 h after treatment, apoptosis was evaluated by DAPI staining and fluorescence microscopy. The percentage of apoptotic cells was determined as described in the text. C, Fas-mediated apoptosis showed that Fas/FasL was functional in 2008 and in 2008C13 cells. For ligation of Fas, cells were treated with purified Fas monoclonal antibody (50 µg/ml) for 24 h or 48 h. The percentage of Fas-mediated apoptosis was determined as in (B). D, 2008 cells were treated as in (Fig. 4A), total RNA was extracted, and expression of FasL and beta-actin mRNAs was
determined by RT-PCR. E, same as Fig. 4A, except that cells were treated with Fas monoclonal antibodies after SB202190 treatment as indicated.

**Figure 6. Induction of caspase activity and cytochrome c release by CDDP.** A, The caspase inhibitor zVAD-fmk prevented CDDP-mediated cell death without affecting CDDP-induced FasL transcription. CDDP-induced apoptosis was caspase-dependent in chemosensitive 2008 cells. Cells were treated with 20 µM of CDDP in the presence or absence of 50 µM of zVAD-fmk as indicated. Cells were then harvested at the indicated times, cell lysates were prepared, and PARP cleavage was examined by immunoblotting with an anti-PARP monoclonal antibody. B, CDDP-induced apoptosis was inhibited by zVAD-fmk. Cells were treated as in A, and the percentage of cells exhibiting apoptotic features was determined. C, CDDP-related FasL mRNA induction was insensitive to zVAD-fmk. Cells were treated as in A, and FasL and beta-actin mRNAs were examined by RT-PCR. D, Time course and release of cytochrome c in the CDDP-sensitive cell line 2008 and its resistant variant 2008C13. Cells were collected at the indicated time points after a 1-h exposure to CDDP, and cytosolic and mitochondrial fractions were subjected to western blot analysis with a monoclonal antibody to cytochrome c (upper panel). The blot was reprobed with an anti-actin antibody to evaluate the loading of the extracts (lower panel).

**Figure 7. Specific activation of JNK and p38 by MKK7 and MKK3 in ovarian carcinoma cells leads to Fas ligand induction and induces 2008C13 apoptosis.** CDDP-resistant 2008C13 ovarian carcinoma cells were infected with recombinant adenovirus vectors encoding activated MKK7 (Ad-MKK7D), MKK3 (Ad-MKK3bE), or GFP (Ad-GFP) as indicated. A, Cell lysates were assayed for JNK or p38 activation by western blot analysis. B, Nuclear fragmentation in 2008C13 cells after infection. Cells were fixed, and nuclear condensation was analyzed with DAPI. C, Chemoresistant 2008C13 cells were treated as in (B), total RNA was extracted, and expression of FasL and beta-actin mRNAs was determined by semi-quantitative RT-PCR.
Figure 8. Proposed model of CDDP-induced signaling pathways leading to JNK activation and apoptosis. Exposure of ovarian carcinoma cells to CDDP caused activation and potent phosphorylation of JNK/p38 resulting in phosphorylation of c-Jun and ATF-2. In the CDDP-sensitive cell line 2008, the persistence of this activation led to the activation of the AP-1 target gene FasL. Release of FasL triggered apoptosis through its receptor Fas, which activates specific caspases, and key substrate were cleaved. Protection against cell death was conferred either by overexpression of a c-Jun mutant lacking the JNK phosphoacceptor sites or by a chemical inhibitor (SB202190) of p38 and JNK that inhibits FasL induction. Inhibition of Fas/FasL interaction through a neutralizing anti-FasL antibody (NOK-2) protected the chemosensitive 2008 cells from apoptosis. In the CDDP-resistant 2008C13 cells, the protection against CDDP-induced apoptosis was presumably due to rapid inactivation of the JNK/p38 activation of MAP kinase phosphatases.
Fig. 1B,C

B

![Bar graph showing the percentage of apoptotic cells over time](image)

C

| Time (h) after 1h exposure | 2008 | 2008 C13 |
|---------------------------|------|----------|
| 0                         | ![Casp-3 32 kD](image) | ![Casp-3 32 kD](image) |
| 6                         | ![Casp-3 17 kD](image) | ![Casp-3 17 kD](image) |
| 12                        | ![Casp-3 17 kD](image) | ![Casp-3 17 kD](image) |
| 18                        | ![Casp-3 17 kD](image) | ![Casp-3 17 kD](image) |
| 24                        | ![Casp-3 17 kD](image) | ![Casp-3 17 kD](image) |
| 48                        | ![Casp-3 17 kD](image) | ![Casp-3 17 kD](image) |

- Casp-3
- PARP
- β-actin
Fig. 2

A

2008

Time: NT 1 3 5 h

GST-cJun (1-79)

Activity (%) - 100 60 60

2008C13

Time: NT 1 3 5 h

GST-cJun (1-79)

Activity (%) - 100 5

B

2008

Time: 0 1m 1h 3h 5h 8h 12h

P-JNK

JNK

P-c-Jun73

c-Jun

P-p38

P-ATF-2

Time: 0 1m 30m 1h 3h 5h 8h 12h 16h

P-Erk

2008C13

Time: 0 1m 1h 3h 5h 8h 12h

P-JNK

JNK

P-c-Jun73

c-Jun

P-p38

P-ATF-2

Time: 0 1m 30m 1h 3h 5h 8h 12h 16h

P-Erk
A

2008

Control

CDDP

SB

SB + CDDP

% Apoptosis

CDDP: -- + -- +
SB: -- + + +
Fig. 4 B.C

**B**

**Counts**

- Untreated: 4%
- CDDP: 51%
- SB: 5%
- SB + CDDP: 9%

**DNA content**

**C**

**FITC**

- HA-c-Jun wt

**DAPI**

- HA-c-Jun(A63/73)

**% HA+/apoptotic cells**

- HA-c-Jun wt: 298 ± 30
- HA-c-Jun(A63/73): 317 ± 20
Fig. 5A-C

A

B

C

- CDDP (μM): 0 20 40 0 20 40 0 20 40
- Time (h): 6 18 24 6 18 24 6 18 24 6 18 24

- Fasl
- Fas
- β-actin

- 2008C13

- Fas Ab (50 ng/ml)
- Control

- 2008C13

- CDDP + Ig
- CDDP + NOK-2

- Apoptosis %
- Time (h)
Fig. 5D,E

**D**

| SB | CDDP | FasL | β-actin |
|----|------|------|---------|
| -  | -    |      |         |
| +  | -    |      |         |
| -  | +    |      |         |
| +  | +    |      |         |

**E**

![Graph showing % Apoptosis](image-url)
Fig. 6A-C

A

| Time (h) | CDDP | z-VAD (50 μM) + CDDP |
|----------|------|----------------------|
| 0        |      |                      |
| 18       |      |                      |
| 24       |      |                      |

(B) Apoptotic cells (%)

- CDDP: -  +  +  +  + z-VAD

(C)

| Time (h) | CDDP | z-VAD (50 μM) + CDDP |
|----------|------|----------------------|
| 0        |      |                      |
| 6        |      |                      |
| 18       |      |                      |
| 24       |      |                      |

Gene expression analysis:
- Fas-L
- β-actin
Fig. 6D

D

|     | Cont. | 6 h | 12 h | 18 h | 24 h |
|-----|-------|-----|------|------|------|
| M   |       |     |      |      |      |
| C   |       |     |      |      |      |

2008

- CYT-C
- β-actin

2008C13

- CYT-C
- β-actin
Fig. 7

A

| Group          | P-JNK | Total JNK | P-p38 | Total p38 |
|----------------|-------|-----------|-------|-----------|
| Control        |       |           |       |           |
| Ad-GFP         |       |           |       |           |
| Ad-MKK7D       |       |           |       |           |
| Ad-MKK3bE      |       |           |       |           |

B

| Group          | Apoptosis |
|----------------|------------|
| Ad-GFP         | 3%         |
| Ad-MKK7D       | 66%        |
| Ad-MKK3bE      | 54%        |

C

| Group          | Fas-L | β-actin |
|----------------|-------|---------|
| Control        |       |         |
| Ad-GFP         |       |         |
| Ad-MKK7D       |       |         |
| Ad-MKK3bE      |       |         |
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J. Biol. Chem. published online March 12, 2003

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