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Paclitaxel (Taxol)-induced Gene Expression and Cell Death Are Both Mediated by the Activation of c-Jun NH_2-terminal Kinase (JNK/SAPK)*

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Paclitaxel (Taxol) is a novel anti-cancer drug that has shown efficacy toward several malignant tumors, particularly ovarian tumors. We reported previously that paclitaxel can induce interleukin (IL)-8 promoter activation in subgroups of ovarian cancer through the activation of both AP-1 and nuclear factor κB. Further analysis of paclitaxel analogs indicates that the degree of IL-8 induction by analysis correlates with the extent of cell death; however, IL-8 itself is not the cause of cell death. This suggests that pathways that lead to IL-8 and cell death may overlap, although IL-8 *per se* does not kill tumor cells. To decipher the upstream signals for paclitaxel-induced transcriptional activation and cell death, we studied the involvement of protein kinases that lead to the activation of AP-1, specifically the c-Jun NH_2-terminal kinase (JNK1), p38, and the extracellular signal-regulated kinase 1 (ERK1). The role of IκB in paclitaxel-induced cell death was also analyzed. Paclitaxel activated JNK, and to a lesser degree p38, but not ERK1. Paclitaxel-induced IL-8 promoter activation was inhibited by dominant-inhibitory mutants of JNK, p38, and the super-repressor form of IκBα, but not by dominant-inhibitory forms of ERK1. Dominant-inhibitory mutants of JNK1 also greatly reduced paclitaxel-induced cell death, and the kinetics of JNK induction was closely followed by DNA fragmentation. These results indicate (i) that paclitaxel activates the JNK signaling pathway and (ii) that JNK activation is a common point of paclitaxel-induced gene induction and cell death.

Paclitaxel (Taxol) is a new generation of chemotherapeutic drug that is effective against malignant non-small cell lung (1, 2), prostate (3, 4), and breast cancer (5), with the most encouraging effects observed in cancer chemotherapy-refractory ovarian cancer (6). Paclitaxel exhibited significant antitumor activity against human ovarian cancer in a nude mouse model (6). Furthermore, paclitaxel significantly inhibited the angiogenic response induced by tumor cell supernatant in mice (7, 8). The primary mechanism of action of paclitaxel is attributed to its ability to bind microtubules and to prevent their disassembly. However, the effect of paclitaxel exceeds that of conventional microtubule-disrupting agents, and there is increasing evidence that paclitaxel has multiple cellular effects in addition to the blockage of mitosis (9–15).

One of the effects of paclitaxel is to alter gene expression. In murine macrophages, paclitaxel can induce the expression of a series of lipopolysaccharide-inducible cytokines, such as IL[1]-1α, IL-1β, TNF-α, and interferon-inducible protein 10 (9–12, 16). In human monocytes, paclitaxel also induces cytokine synthesis (17). Most pertinent to this report, paclitaxel can induce IL-8 gene expression at the transcriptional levels in subsets of human ovarian cancer lines (14). This induction is mediated by the activation of NF-κB and AP-1 transcription factors, which bind to cognate sites in the IL-8 promoter. Gel shift assays show that paclitaxel caused a marked increase in protein binding to AP-1 and NF-κB cognate sequences in paclitaxel-responsive cells but not in nonresponsive cells (15). However, little is known about the upstream signaling events that lead to the activation of these transcription factors in response to paclitaxel. The present study identifies upstream signals that are activated by paclitaxel and mediate its effects. Identification of upstream signals involved in IL-8 gene induction may have broad ramifications because a tight correlation between the ability of paclitaxel analogs to induce IL-8 and cell death among ovarian tumors has been observed previously (18). It is important to determine if the same signals that induce IL-8 gene induction also induce cell killing.

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases that mediate numerous types of extracellular stimuli (19, 20). The ERK member of MAPK is activated by growth factor via a Ras-dependent signal transduction pathway (21). ERK can phosphorylate and activate various transcription factors, including c-Myc and TCF/Elk1 (22). In contrast, the JNK (JNK1 and JNK2) members of MAPKs, also designated stress-activated protein kinases (SAPKs), can be activated by proinflammatory cytokines (23–25) and environmental stress such as UV light (26, 27), γ-irradiation (26), heat shock, osmotic shock (28), shear stress (29), growth factor withdrawal (30), ceramide (31), and protein synthesis inhibitor (23). The activation of the JNK cascade is also observed in cells stimulated by various mitogenic factors such as growth factors, oncogenic Ras, phorbol ester, and T-cell activation signaling (32, 33). Like MAPK, JNK activation requires phosphorylation

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[1] The abbreviations used are: IL, interleukin; TNF, tumor necrosis factor; NF-κB, nuclear factor κB; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; SAPK, stress-activated protein kinases; MEKK, mitogen-activated protein kinase kinase; GST, glutathione *S*-transferase; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase; PBS, phosphate-buffered saline; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; IκB, inhibitor of NF-κB.

at two conserved residues, threonine and tyrosine, by the MAP kinase kinase SEK1/MKK4/JNK kinase (34). The latter is in turn phosphorylated by upstream kinase MAPK kinase kinase 1 (MEKK1) (32, 35). The JNK kinase cascade was shown to be a common pathway involved in cell proliferation and stress-response signaling. There are at least three major transcription factors that can be phosphorylated by JNK: c-Jun (36), ATF-2 (37), and TCF/Elk1 (38).

In this report we demonstrate that JNK is strongly activated by paclitaxel, a chemotherapeutic agent. The activation of JNK occurs in a dose- and time-dependent manner. The inhibition of JNK activity greatly reduced paclitaxel-induced IL-8 gene expression and cell death, whereas the inhibition of p38 produced a smaller but noticeable effect. These results show the involvement of JNK and p38 in paclitaxel-induced IL-8 transcription activation and in mediating cell death, both of which have anti-tumor affects.

MATERIALS AND METHODS

Cells, Antibodies, and Reagents—The human ovarian cancer cell line OVCA 420 (a gift from Dr. Robert Bast, Jr., M. D. Anderson, Houston, TX) was maintained as monolayer cultures in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 5% fetal bovine serum. The derivation of these cells has been described previously (39). Rabbit anti-JNK1, anti-ERK1, and anti-p38 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The monoclonal antibody M2 was obtained from IBI-Kodak (New Haven, CT). Anti-c-Jun and anti-c-Fos were purchased from Santa Cruz Biotechnology. Myelin basic protein (Bio-Rad) was prepared at a concentration of 5 μg/μl in kinase reaction buffer.

Plasmids and Recombinant Proteins—GST-c-Jun (1–79) linked to Sepharose beads was used as the substrate (40). The pcDNA3-FLAG-JNK1(APF) and pCMV-FLAG-p38(AGF) were kindly provided by Dr. R. J. Davis (University of Massachusetts, Worcester (25, 41). pCMV IκBα and control empty vector (pCMV4) were generous gifts from Dr. A. S. Baldwin Jr. (University of North Carolina, Chapel Hill) (42, 43). pCMVERK1 was a generous gift from Dr. D. A. Brenner (University of North Carolina, Chapel Hill) (44). IL-8 −133 CAT was described previously (15).

Paclitaxel Treatment—Cells were seeded in 100-mm plates (Falcon Inc., Plymouth, U. K.) and grown in Dulbecco's modified Eagle's medium supplemented with 10% endotoxin-free fetal bovine serum until the cells were 60–75% confluent. Immediately before treatment the medium was removed, then cells were washed once with sterile endotoxin-free phosphate-buffered saline (PBS) and treated with paclitaxel or the diluent, Me_2SO stock. Paclitaxel was maintained at −70 °C at a concentration of 30 mM. The final concentration of Me_2SO never exceeded 0.1%.

Transfection and CAT Assay—Cells from the human ovarian cancer OVCA 420 cells were transfected using LipofectAMINE reagent (Life Technologies, Inc.) (45). The transfection mixture was removed 6 h after treatment. The cells were incubated in complete medium for 6 h and then treated with Me_2SO or paclitaxel for 24 h. Cell extract was prepared, and CAT enzymatic activity was assayed as described elsewhere (46, 47). To address variations in transfection efficiency, each construct was tested using at least two separate plasmid preparations in three independent experiments. These multiple experiments were intended to eliminate experiment-to-experiment variations in transfection efficiency.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays—Nuclear extracts from OVCA 420 cells were prepared as described previously (48), with slight modifications. Electrophoretic mobility shift assays were performed as described previously (49, 50). 4–6 μg of nuclear extract was incubated with 2 μg of poly(dI-dC) and 50,000–100,000 cpm of ^{32}P end-labeled DNA probe in a 20-μl reaction volume. After incubation for 20 min at room temperature, the complex was subjected to electrophoresis in a 6% nondenaturing polyacrylamide gel containing 1× Tris-glycine. Antibody supershift experiments were performed by preincubating 1 μg of antibody for 2 h on ice with each binding reaction before the addition of the DNA probe.

Immunoprecipitation and Western Blot—The cell extract from OVCA 420 cells that were transfected with mutant kinase-expressing vector was prepared in 1 ml of RIPA lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM NaF, 0.2 mM Na_3VO_4, 100 units/ml aprotinin). The cell lysates were incubated on ice for 10 min. Cellular debris was pelleted at 3,000 rpm at 4 °C for 15 min. The cell extracts were immunoprecipitated with anti-FLAG (5 μg/ml) antibody and then analyzed by immunoblotting with anti-JNK1 (1.2 μg/ml), anti-p38 (1.2 μg/ml), and control antibodies using standard techniques (51).

Photometric Sandwich ELISA for Cytoplasmic Histone-associated DNA Fragments—10^5 cells were plated onto 24-well plates the day before stimulation. Cells were incubated with paclitaxel for different time intervals. The cells were harvested and treated with lysis buffer. The lysate was separated into cytoplasmic and nuclear fractions by centrifugation. 20 μl of the supernatant, which contained the cytoplasmic fraction, was used to measure DNA fragmentation and histone release from the nucleus. The measurement was performed according to the manufacturer's protocol for the Cell Death Detection ELISA^{plus} kit (Boehringer Mannheim). Briefly, the supernatants were placed in streptavidin-coated microtiter plates followed by the addition of biotin-labeled anti-histone and peroxidase-conjugated anti-DNA antibodies. The anti-histone antibody, bound to the plate via biotin-streptavidin, also bound histones from released nucleosomes. The anti-DNA antibodies recognized DNA in the nucleosomes and gave a colorimetric reaction upon the addition of a photometric substrate, 2,2′-azino-di[3-ethylbenzthiazolin-sulfonate] (52, 53).

Transient Transfection and LacZ Cell Death Assay—OVCA 420 cells were plated 24 h before transfection at a density of 2×10^3 cells/well in a 12-well plate. Cells were cotransfected with the pCMV-βgal (0.1 μg) plasmid, which expresses galactosidase and the plasmids containing the super-repressor IκBα, dominant-inhibitory mutant kinase (1 μg), or a control vector using the SuperFect transfection reagent (Qiagen, Valencia, CA). Transfections were performed in duplicate. 6 h after transfection, the cell medium was replaced with fresh complete medium for 12 h, and then the cells were treated with 30 μM paclitaxel or Me_2SO. After 24 h of treatment, cells were harvested, washed, and fixed in 0.5% glutaldehyde in PBS. These cells were washed twice with PBS, resuspended in staining solution containing PBS (pH 7.4), 1 mM $MgCl_2$, 10 mM $K_4Fe(CN)_6$, 10 mM $K_3Fe(CN)_6$, and 1 mM X-gal (added just before use) for 1–3 h, and washed twice with PBS. The β-galactosidase-positive cells in each well were counted. Cell survival was determined as the number of blue cells in paclitaxel-treated group/number of blue cells in Me_2SO-treated group × 100%.

Solid Phase and Immunocomplex Kinase Assay—After paclitaxel or Me_2SO treatment, the cells were washed with ice-cold PBS and lysed with 900 μl of low salt buffer (150 mM NaCl, 20 mM Tris, pH 7.5, 5 mM EDTA, 1% Triton X-100, 50 mM NaF, 10% glycerol, 1 mM Na_3VO_4, 4 μg/ml aprotinin, 20 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin). The lysate was mixed in 4 °C for 15 min and centrifuged at 12,500 rpm for 12 min at 4 °C. The supernatant was transferred to a clean tube, and 50 μg of protein was incubated with 10 μg of GST-c-Jun (1–79) beads for 2 h at 4 °C. The beads were then washed twice with the lysis buffer and twice with the kinase assay buffer (1× kinase assay buffer: 20 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 20 mM β-glycerolphosphate containing 20 mM *p*-nitrophenyl phosphate, 0.1 mM Na_3VO_4, 2 mM dithiothreitol). Kinase reactions were initiated by the addition of 5 μl of 7 × kinase buffer, 0.133 μl of cold 10 mM ATP, 1 μl of [γ-^{32} P]ATP (3,000 Ci/mmol), and H_2O to a final volume of 35 μl, incubated for 20 min at 30 °C, and terminated with 7 μl of 3× SDS sample buffer. Samples were boiled for 5 min, centrifuged at 12,500 rpm for 5 min, and then the supernatant was resolved by 10% SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie Blue and then dried. The phosphorylation of GST-c-Jun was visualized by autography. Immunocomplex kinase assays were carried out as described (54) with some modifications. MAPKs were precipitated by incubation with protein A-agarose beads (Santa Cruz) and specific antibodies (*e.g.* anti-JNK1, anti-p38, or anti-ERK1 antibody) in lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM NaF, 0.2 mM Na_3VO_4, 100 units/ml aprotinin) for 4 h at 4 °C on a rocket platform. Immunoprecipitates were collected by centrifugation at 2,500 rpm for 5 min at 4 °C. The precipitates were washed twice with lysis buffer and twice with kinase assay buffer (50 mM HEPES, pH 7.4, 0.1 mM EDTA, 0.01% Brij 35, 0.1 mg/ml bovine serum albumin, 0.1% β-mercaptoethanol, 0.15 M NaCl) and then mixed with 5 μg of the indicated substrates and 10 μl of ATP mix (930 μl of kinase buffer, 6 μl of 50 μM ATP, pH 7.0, 20 μl of 2.0 M $MgCl_2$, and 44 μl of [γ-^{32} P]ATP at 10 mCi/ml before an incubation for 20 min at 30 °C. The reactions were terminated with SDS sample buffer and boiled for 5 min. The supernatants were resolved by 10% SDS-polyacrylamide gel electrophoresis.

Immunoprecipitation Kinase Assays of p38—The p38 assay was performed according to the manufacturer's instruction for the p38 MAPK assay (New England BioLabs, Beverly, MA). Briefly, after immunopre-

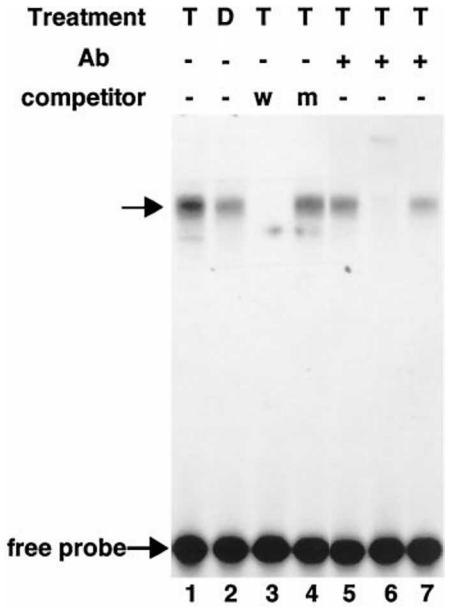

FIG. 1. **Paclitaxel induces AP-1 DNA binding activity.** Nuclear protein extract was prepared from OVCA 420 cells stimulated with paclitaxel (*T*) (30 μM) or Me_2SO (*D*) for 30 min. 4 μg of the extract was mixed with ^{32}P-labeled AP-1 DNA fragment (−130 to −114 segment of the IL-8 gene) in 20 μl of binding reaction buffer in the absence or presence of unlabeled wild type (*w*) DNA fragment or a mutant (*m*) DNA fragment. For the supershift study, 1 μl of antibody, either normal unimmunized serum (*lane 5*), anti-c-Jun (*lane 6*), or anti-c-Fos (*lane 7*) was incubated with nuclear extracts and reaction buffer on ice for 1 h before the addition of the radiolabeled probe. The *arrow* indicates the position of the AP-1 DNA-protein complex.

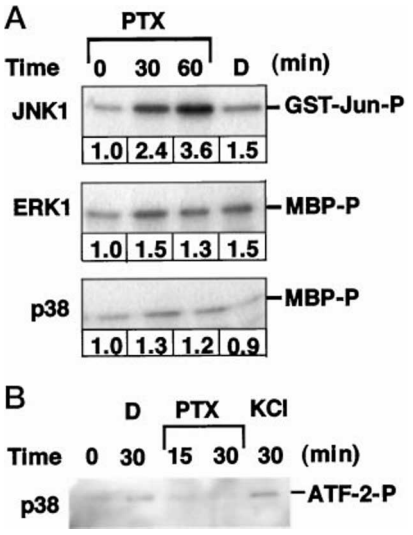

FIG. 2. **Paclitaxel activates JNK.** *Panel A*, paclitaxel (*PTX*) treatment affects JNK1. Human OVCA 420 cells were treated with 30 μM paclitaxel for 0, 30, and 60 min or Me_2SO (*D*) for 30 min. Treated cells were subsequently harvested, and cell lysates were prepared. Endogenous JNK1, ERK1, and p38-MAPK activities (*top*, *middle*, and *bottom*, respectively) were determined by immunocomplex assays using GST-c-Jun (1–79) as JNK substrates and myelin basic protein (*MBP*) as ERK1 and p38-MAPK substrates as designated at the *right*. *Panel B,* paclitaxel failed to activate p38-MAPK. OVCA 420 cells were treated with 30 μM paclitaxel for 15 and 30 min, with 700 mM KCl for 30 min, or with Me_2SO (*D*) for 30 min. p38-MAPK activity was measured by Western blotting of phospho-ATF-2 as described under "Material and Methods." *Numbers* under the *bands* indicate the fold activation of JNK, ERK, or p38 as quantitated by video image densitometry. The autoradiogram shown is from one representative experiment. This experiment was repeated three times with comparable results.

cipitation of p38 MAPK with anti-p38 antibody (1:50 to 1:100 dilution), the immunocomplex was incubated with ATF-2 and 200 μM cold ATP in kinase buffer. Kinase reactions were incubated at 30 °C for 15 min and terminated with SDS sample buffer. The products were resolved by 10% SDS-polyacrylamide gel electrophoresis. Samples were analyzed by a Western blot using a phospho-specific ATF-2 antibody (1:1,000 dilution). Protein bands on immunoblots were visualized by enhanced chemical luminescence (Amersham Pharmacia Biotech).

RESULTS

Paclitaxel Activates AP-1 Binding Activity—In a previous report, we showed that paclitaxel activates DNA binding through the AP-1 factor in human OVCA 420 cells (15). The results are confirmed as shown in Fig. 1, *lanes 1* and *2*. Paclitaxel-induced binding was specifically competed by an excess of wild-type unlabeled AP-1 target site but not by an oligonucleotide containing point mutations, which resulted in the loss of AP-1 binding (*lanes 3* and *4*). Anti-c-Jun and anti-c-Fos antibodies were used in supershift assays. The results show that an anti-c-Jun antibody specifically diminished this DNA-protein complex, but a control normal rabbit serum had no effect (*lanes 5* and *6*). Antibody directed against c-Fos protein had a small effect and decreased binding to the level of the Me_2SO control reproducibly. Results from the cold competition and supershift assays indicate that the DNA-protein complex formed in response to paclitaxel contained c-Jun and probably c-Fos (Fig. 1).

JNK1 Is Activated by Paclitaxel—Because paclitaxel activates c-Jun but JNK1 activates and phosphorylates c-Jun, the effect of paclitaxel on JNK activation is worthy of investigation. A kinetics analysis of kinase activation by paclitaxel was first performed. The activities of JNK, ERK, and p38 in the cell lysates were determined by an immunocomplex kinase assay as described under "Materials and Methods." As shown in Fig. 2*A*, the activation of JNK1 activity was observed at the earliest measured time point of 30 min at a concentration of 30 μM paclitaxel. Importantly, this time course is coincidental with the transcriptional activation of the IL-8 gene by paclitaxel (14). p38-MAPK and ERK1 activity increased slightly (<1.5 fold) (Fig. 2*A*). Others have encountered difficulties in observing p38 activation.[2] To exclude the possibility that the absence of a significant p38 activation by paclitaxel was caused by nonfunctional antibody or substrate, an activity assay was performed. A specific antibody to p38 MAPK was used to immunoprecipitate p38 from cell lysates selectively. The resulting immunoprecipitate was incubated with a preparation of ATF-2 fusion protein in the presence of cold ATP and kinase buffer. The phosphorylation of ATF-1 at Thr-71 was measured by Western blotting using a phospho-specific ATF-2 (Thr) antibody. Paclitaxel treatment did not increase ATF phosphorylation relative to the Me_2SO control as detected by this assay. The positive control consists of KCl-activated cells (Fig. 2*B*, *far right lane*). This indicates that the small increase in p38 observed in Fig. 2*A* is not caused by the assay conditions, but likely reflects the small magnitude of response.

JNK Activation Occurs at a Low Concentration of Paclitaxel—Experiments were performed to determine if paclitaxel activates JNK in a dose-dependent manner. The results show that the activation of JNK1 activity to varying magnitude (4.0–6.0-fold) was observed with as little as 0.1 μM paclitaxel (Fig. 3*A*). This level of paclitaxel which induced JNK is easily obtainable during chemotherapy. To extend the dose-response analysis, lower concentrations of paclitaxel were used (Fig. 3*B*), but these did not activate JNK. In contrast, ERK1 was not strongly activated by paclitaxel. A slight induction of p38 was reproducibly seen at a concentration of 3 μM paclitaxel in three experiments (Fig. 3*A*). The concentration of paclitaxel required

[2] S. E. Earp, personal communications.

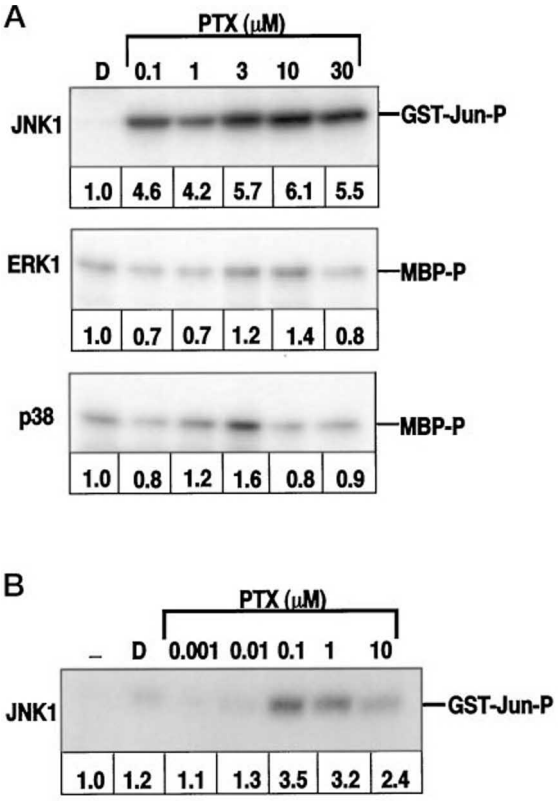

FIG. 3. **JNK is induced by low concentrations of paclitaxel.** *Panel A*, OVCA 420 cells were treated with different concentrations of paclitaxel (*PTX*) as indicated or Me_2SO (*D*) for 30 min. Endogenous JNK1, ERK1, and p38-MAPK activities (*top*, *middle*, and *bottom*, respectively) were determined by immunocomplex assays with the substrates indicated at the *right*. *Panel B*, JNK activation by different concentration of paclitaxel in OVCA 420 cells. *Numbers* under the *bands* are as in Fig. 2. see Fig. 2 legend. The autoradiogram shown is from one representative experiment; the experiment was repeated three times with comparable results.

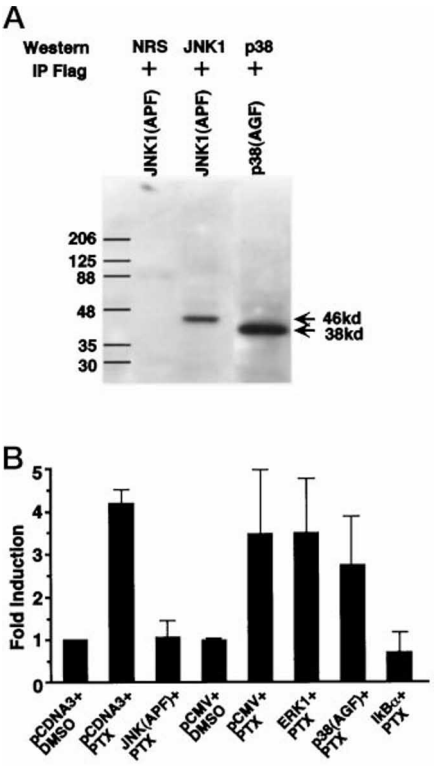

FIG. 4. *Panel A*, protein expression by dominant-inhibitory JNK and p38-MAPK mutants. Cells were transfected with pcDNA3-Flag-JNK(APF) or pCMV-Flag-p38(AGF) as indicated. Transient transfectants were immunoprecipitated by using mouse monoclonal antibody to the Flag epitope (M2), and then the immunoprecipitates were examined by Western blotting using normal rabbit serum, JNK, and p38 antibody, respectively. The *arrows* indicate the 46-kDa JNK1 and the 38-kDa mutant p38 protein detected by JNK- and p38-specific antibodies respectively, whereas normal rabbit serum showed no specific bands. *Panel B*, IL-8 promoter activation requires JNK and NF-κB. Cells were cotransfected with a IL-8--133CAT (5 μg) reporter and dominant-inhibitory mutant kinase expressing vectors or the super-repressor form of IkBα (10 μg) as indicated. The pCDNA3 empty vector served as a control for the JNK(APF) plasmid and the pCMV empty vector as a control for the ERK1, p38, and IkBα plasmids. After transfection, OVCA 420 cells were then treated with Me_2SO or 30 μM paclitaxel (*PTX*) for 24 h as indicated. Cell extracts were prepared, and CAT assays were performed as described under "Materials and Methods." These results represent the average ± S.E. of three independent experiments.

to activate JNK is lower than that required to induce IL-8 gene expression (previously observed at 5–30 μM paclitaxel). This suggests that paclitaxel can induce JNK activation efficiently and that the induction of other factors necessary for IL-8 transcription may require a higher concentration of paclitaxel.

Dominant-inhibitory Mutants of JNK Blocked IL-8 Promoter Activation by Paclitaxel—To assess directly the involvement of JNK or p38 in IL-8 promoter activation by paclitaxel, dominant-inhibitory mutants were used. OVCA 420 cells were transfected with FLAG-tagged dominant-inhibitory mutants of JNK and p38 (pcDNA3-FLAG-JNK1(APF) and pCMV-Flag-p38(AGF)) (25, 41). Transient transfected cell lysates were immunoprecipitated with mouse monoclonal antibody to Flag (M2) to verify the expression of dominant-inhibitory mutants. The immunoprecipitates were examined by Western blotting with normal rabbit serum, anti-JNK, or p38 antibodies. As shown in Fig. 4*A*, transfection of the dominant-inhibitory mutant of JNK resulted in the expected expression of a 46 kDa protein, whereas transfection of the dominant-inhibitory mutant of p38 produced a 38-kDa mutant protein; normal rabbit serum did not precipitate any specific bands (Fig. 4*A*).

The ability of these two mutants to disrupt JNK and p38-MAPK signaling pathway has been reported extensively in the literature and is well characterized (25, 26, 41, 55). To determine if these dominant-inhibitory mutants can block paclitaxel-induced IL-8 promoter activity, an IL-8–133 CAT reporter construct was cotransfected (15) with one of the following dominant-inhibitory mutants: ERK1, JNK(APF), p38(AGF), or the appropriate empty control vectors (pcDNAs or pCMV). As reported previously, paclitaxel increased IL-8 promoter activity significantly over the Me_2SO control; the introduction of the empty vector (pcDNA3) did not affect this induction (Fig. 4*B*, *first two lanes* from *left*). Overexpression of a dominant-inhibitory mutant of JNK1 subcloned in the pcDNA3 vector greatly reduced activation of the IL-8 CAT reporter by paclitaxel (*third lane*). In the second part of the same experiment, cells were transfected with an empty control vector, pCMV, and treated with paclitaxel. This control is necessary because the dominant-inhibitory mutants of ERK1 and p38 were subcloned in the pCMV vector. Again, paclitaxel induced IL-8 promoter activation (*fourth* and *fifth* lanes). The cotransfection of the dominant-inhibitory mutants of ERK1 had little effect on IL-8 activation; p38 dominant-inhibitory mutant genes had a more noticeable effect on IL-8 activation. The super-repressor IκBα abolished all induction of the IL-8 CAT construct. This indicates that both the JNK-mediated activation of AP-1 and NF-κB activation by paclitaxel are important in IL-8 gene induction by the drug.

Paclitaxel-induced Activation of JNK Correlates with Cell Death—Previous studies suggest a model in which persistently activated JNK activity is required for inducing apoptosis. To

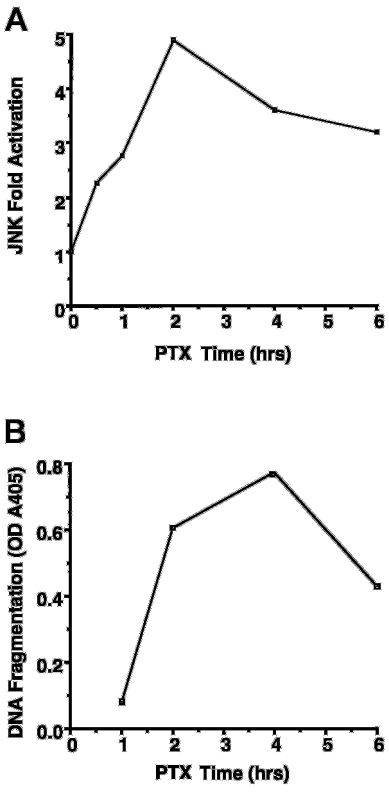

FIG. 5. **Time course of JNK activation and DNA fragmentation in human OVCA 420 cells after treatment with paclitaxel (*PTX*).** *Panel A*, OVCA 420 cells were treated with 1 μM paclitaxel for 30 min, 1 h, 2 h, 4 h, or 6 h. JNK activities were analyzed by the immunocomplex kinase assay. The fold activation of JNK was calculated by comparison with basal JNK activities in Me_2SO-treated controls. Data shown represent the mean values from two independent experiments. *Panel B*, OVCA 420 cells were incubated with paclitaxel for the indicated time. Cell death was measured by the photometric ELISA assay for cytoplasmic histone-associated DNA (see "Materials and Methods"). The results represent the mean value from three independent experiments.

determine if this is the case, longer kinetics analysis was performed to determine if a connection between rises in JNK activity and DNA fragmentation could be observed. As shown in Fig. 5, paclitaxel activated JNK within 30 min of treatment and reached a maximum level at 2 h. The level then declined slightly between 4 and 6 h (Fig. 5*A*). The kinetics of histone-associated DNA fragmentation as determined by ELISA (see "Materials and Methods") followed that of JNK activation. Paclitaxel induced significant cell death 2 h after treatment and reached a peak at 4 h, decreasing at 6 h (Fig. 5*B*). These results suggest that JNK activation precedes cell death and may play a critical role in paclitaxel-induced cell death. To determine if JNK is directly involved in cell death by paclitaxel, dominant-inhibitory mutants of JNK were used in the following experiments.

Dominant-inhibitory Mutants of JNK1 and MEKK1 Suppress Paclitaxel-induced Cell Death—A previous report studied a panel of 12 paclitaxel analogs and noted a striking correlation between the induction of IL-8 and cell death (18). We hypothesize that the pathways leading to IL-8 gene activation may also lead to cell death. Many reports have shown that JNK activation is involved in apoptosis caused by UV, γ-irradiation (26, 27), growth factor withdrawal (30), ceramide treatment (31), or other anti-cancer drugs (55–57). Here, we examined the role of JNK in paclitaxel-induced cell death.

To determine whether JNK signaling contributes to paclitaxel-induced cell death, a β-galactosidase expression assay was used to measure cell viability in cells transfected with a dominant-inhibitory mutant that blocked JNK signaling. OVCA 420 cells were cotransfected with pCMV-βgal and a plasmid containing either a dominant-inhibitory kinase gene or an empty vector. Each transfection was performed in duplicate. The β-galactosidase-positive cells (blue) were stained after a 24-h exposure to paclitaxel and then counted by microscopic examination. β-Galactosidase-positive signal was used to measure the number of viable cells capable of producing the gene product. Overexpression of the dominant-inhibitory mutants of JNK1 (JNK1 (APF)) blocked paclitaxel-induced cell death by approximately 80% (Fig. 5, *A* and *B*). Dominant-inhibitory forms of MEKK1, which activates the JNK activator SEK1, also decreased paclitaxel-induced cell death (Fig. 5*B*). Transfection of the dominant-inhibitory mutant p38 ((p38(AGF)) resulted in a small inhibition of cell death induced by paclitaxel. Because NF-κB is activated by paclitaxel (15), it is relevant to assess if molecules in the NF-κB pathway contribute to paclitaxel-induced cell death. Although NF-κB is generally thought of as a survival factor that prevents cell death (42, 58, 59), there is also contrary evidence (60–62). Additional experiments from another laboratory reveal that the super-repressor IκB form did not sensitize cells to paclitaxel-induced death,[3], but it did sensitize cells to TNF-α, a DNA-damaging agent, and another chemotherapy (42). Fig. 6 shows that the super-repressor form of IκBα as well as the empty vector also did not suppress paclitaxel-induced cell death. Thus more extensive studies are necessary to clarify the role of NF-κB in paclitaxel-induced cell death.

DISCUSSION

A previous report from our group indicates that paclitaxel specifically induces the transcription of the IL-8 gene via activation of its promoter (14). This occurs in approximately half of ovarian tumor cell lines and, significantly, in half of freshly explanted tumors. The induction is by the transcriptional activation of IL-8 promoters. The paclitaxel-responsive regulatory elements in the IL-8 promoter consist of the AP-1 and NF-κB cognate binding sites (15). Further analysis shows that both the AP-1 and NF-κB transcription factor are activated by paclitaxel. Gel shift analysis shows that paclitaxel treatment of ovarian tumors causes increased binding to an AP-1 *cis*-acting regulatory element in the IL-8 promoter (15). This is correlated with enhanced activation of the IL-8 promoter as well as with the activation of a promoter with the canonical AP-1 binding site. These findings provide a venue to examine intracellular events that lie upstream of AP-1 which are activated by paclitaxel. Once these intracellular events are identified, it is then feasible to determine if they contribute to the therapeutic effects of paclitaxel in controlling tumor growth and proliferation. The activation of these intracellular events in subsets of tumor cells may also provide a prognostic marker that can predict the efficacy of paclitaxel in controlling tumors *in vivo*.

In this report, we assess whether MAPK, ERK1, JNK1, and p38 constitute upstream signaling events that lead to the transcriptional induction of IL-8 by paclitaxel. *In vitro* kinase assays show that JNK can be activated by paclitaxel. The activation of JNK by paclitaxel suggests its potential involvement in IL-8 induction because activated JNK is known to phosphorylate several transcription factors, including c-Jun, ATF2, and TCF/Elk1 (32, 37, 38). Indeed, the JNK1 dominant-inhibitory mutant blocked paclitaxel-induced IL-8 promoter activity (Fig. 4). These results support the conclusion that JNK-mediated activation of c-Jun leads to IL-8 gene activation. The induction of IL-8 has important biologic consequences in controlling tu-

[3] C.-Y. Wang, personal observation.

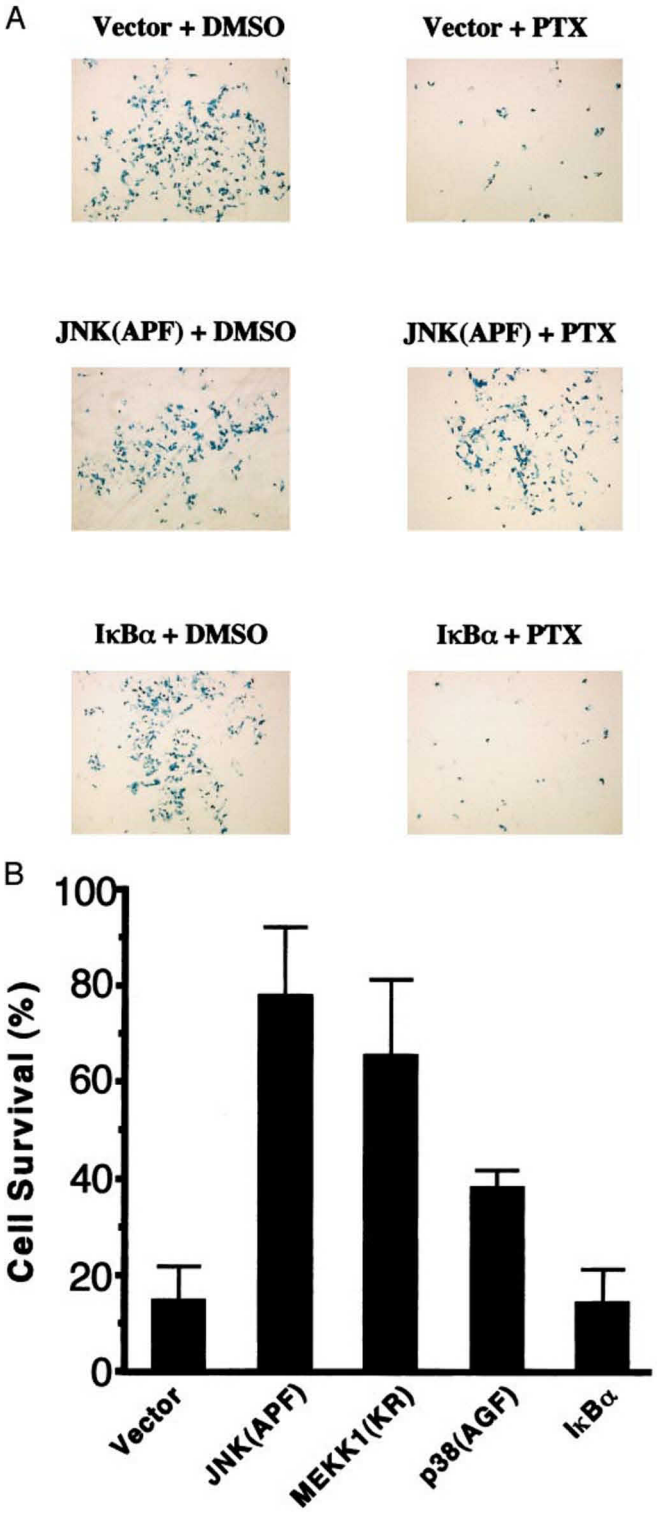

FIG. 6. **Dominant-inhibitory JNK1 and MEKK1 mutants block paclitaxel-induced cell death.** *Panel A*, OVCA 420 cells were co-transfected with pCMVβ-Gal (0.1 μg/12-well plate) and either an empty expression vector control or with vector encoding mutant kinase or IkBα (1 μg). After 6 h of transfection, the medium was replaced with fresh complete medium, and the cells were incubated for 12 h and then treated with 30 μM paclitaxel (*PTX*) or Me_2SO (*DMSO*). After 24 h of treatment, cells were fixed and stained with X-gal, and β-galactosidase-positive cells in each well were counted. Cell survival was determined by the percentage of blue cells in the paclitaxel-treated group divided by the percentage of blue cells in Me_2SO-treated group multiplied by 100%. The results shown here represent the mean ± S.D. of three independent experiments performed in duplicate. *Panel B*, X-gal colorimetric staining of vector-, or JNK(APF), or IkBα-transfected cells treated with paclitaxel or Me_2SO. All panels are magnified × 40.

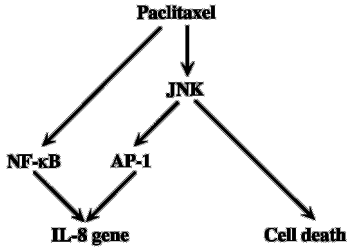

FIG. 7. **The signaling pathway of paclitaxel-induced gene expression and cell death.** The activation of JNK by paclitaxel is a converging point for drug-induced cell death and in the activation of IL-8. Paclitaxel treatment leads to JNK activation, which in turn leads to AP-1 activation. AP-1 together with NF-κB, which is also induced by paclitaxel, result in IL-8 promoter activation. JNK is also involved in cell death induced by paclitaxel as shown by the dominant-inhibitory JNK mutants.

mor growth, as another study from our group shows that the introduction of IL-8 into human ovarian tumors resulted in massive granulocytic and monocytic infiltration and the subsequent reduction or elimination of tumor growth.[4]

Activation of the JNK signaling pathway is increasingly found to play a major role in differentiation and cell death. It is known that JNK1 is activated predominantly in response to stress-activating signals such as growth factor withdrawal, heat shock, osmotic, UV light, protein synthesis inhibitors, and proinflammatory cytokines (24, 25). On the other hand, activation of the JNK pathway can also induce apoptosis or cell death. For example, DAXX, a recently identified Fas-binding protein, can induce apoptosis via activation of the JNK pathway (64). The apoptosis signal-regulated kinase (ASK), is also an activating kinase of the JNK pathway. In response to TNF-α, ASK is sufficient to induce apoptosis and is required for TNF-α-induced apoptosis (65). Several reports have also demonstrated that the duration of JNK activation is a determining factor for cell proliferation or death (26). Transient JNK induction, such as signaling from anti-CD28 plus phorbol 12-myristate 13-acetate (PMA) or PMA plus ionomycin, causes a rapid course of JNK1 activation leading to cell proliferation. More sustained JNK activation (*e.g.* UV light, γ-irradiation, DNA damage, drug treatment) causes cell apoptosis (26, 55, 66). Our results are consistent with the observations reported by Chen and co-workers because paclitaxel, like other stress agents, causes a more sustained pattern of JNK activation (Fig. 5), and this prolonged activation of JNK is directly linked to cell death based on the use of dominant-inhibitory JNK mutants. A worthy line of investigation is to determine if JNK activation occurs in response to microtubule stabilization by paclitaxel or if it is independent of microtubule stabilization (66). It will be intriguing to differentiate these molecular mechanisms of paclitaxel's action. One way to approach this may be the use of paclitaxel analogs. Upon the examination of 12 structurally related paclitaxel analogs, Watson *et al.* (18) reported a tight correlation between paclitaxel-induced gene expression and cell death. In their hands, paclitaxel analogs that most dramatically up-regulated IL-8 expression were the most effective in inhibiting cell survival. They also found that IL-8 production was not directly responsible for cell killing because treatment with anti-IL-8 antibodies failed to block cell death (18). Taken together, all of these studies suggest that JNK activation is required for both IL-8 gene transcription and cell killing.

The p38 kinase pathway that is activated by many stimuli common to the JNK/SAPK pathway is activated to a lesser degree in paclitaxel-treated human ovarian cancer cells (25, 67,

[4] L.-F. Lee, R. P. Hellendall, Y. Wang, J. S. Haskill, N. Mukaida, K. Matsushima, and J. P.-Y. Ting, submitted for publication.

68). Transfection of the dominant-inhibitory mutant p38 (p38(AGF)) resulted in a small inhibition of IL-8 promoter CAT activity and cell death induced by paclitaxel. In composite, these data point to a role for p38 in all of these assays. During the preparation of this manuscript, another report showed that paclitaxel primarily affected JNK activation but not p38-MAPK activation in B lymphoblasts (57). Thus the effect of paclitaxel on p38 is likely slight.

An earlier study also showed that IL-8 induction by paclitaxel requires activation of the NF-κB transcription factor (15). Recent work from several laboratories has demonstrated that the Rel/NF-κB transcription factor regulates apoptosis by serving as a survival signal in many cell types (42, 58, 59). Furthermore, Wang et *al*. (42) have shown that cells transfected with the super-repressor IκBα, which inactivates NF-κB, are more prone to cytotoxicity in response to pro-apoptotics including the inflammatory cytokine TNF-α and the DNA-damaging agents, ionizing radiation and daunorubicin.

Like many other activators of the cell stress response, paclitaxel activates both AP-1 and NF-κB signaling pathways and induces cell death (15). Similar divergent signaling via the TNF-α receptor is well characterized, involving separate activation of cell death pathways (through the TNF-α receptor-associated factor Traf1) and cell survival pathways through the NF-κB-activating factor Traf2 (69). Inhibition of Traf-2-dependent signaling potentiates TNF-α-induced apoptosis (70). Similarly, in addition to its role in AP-1 signaling, the JNK activator MEKK1 is implicated in activation of NF-κB (71–73), although the mechanism of NF-κB activation by MEKK1 remains unclear. Thus, stress signaling involving MEKK1 and TNF-α (and likely paclitaxel and other stress stimulators) appears to involve a balance of death and survival pathways, the final outcome of which may depend on quantitative, combinatorial, or temporal considerations. In support of this, activation of caspases during Fas-stimulated apoptosis blocks subsequent TNF-α-mediated destruction of IκBα and is coincident with complete cleavage of MEKK1 by caspase(s) to a cytosoluble, pro-apoptotic form (63). The dual activation of NF-κB and JNK by paclitaxel may similarly involve a balance between these two signals with likely opposite effects on cell survival. On the other hand, NF-κB-mediated signals that regulate the process of apoptosis are likely complex. For example, under some circumstances, activation of NF-κB may also promote apoptosis (60-62). In one study (61), apoptotic death induced by serum withdrawal was shown to be accompanied by NF-κB activation in a human embryonic kidney cell line. Interestingly, their results show that the transient or stable expression of *bcl-2*, which is known to interfere with apoptosis, reduced κB-dependent reporter gene expression and reduced cell death. Thus the role of NF-κB as a promoter or attenuator of cell death may ultimately depend on both the cell type and the nature of the apoptosis-inducing stimuli.

In addition to the importance of JNK in paclitaxel-induced cell death, we have recently observed that DEVD (Z-Asp-Glu-Val-Asp-fluoromethyl ketone), which is known to inhibit caspase-3/CCP32, and the broad spectrum caspase, inhibitor VAD (Z-Val-Ala-Asp- fluoromethyl ketone), efficiently block paclitaxel-induced histone-associated DNA fragmentation in human ovarian cell lines. However, these caspase inhibitors do not significantly affect JNK activation.[5] These results suggest that the caspase cascade plays a critical role in paclitaxel-mediated apoptosis but may be located downstream of JNK signaling or may represent an additional route of apoptosis.

In summary, this report is the first to show that JNK is directly involved in paclitaxel-induced IL-8 transcription activation and cell death as summarized by the model in Fig. 7. In this model, paclitaxel treatment leads to the activation of JNK, which is important both in the activation of AP-1 and in causing cell death. Paclitaxel also activates NF-κB, which together with AP-1, leads to the activation of IL-8 genes. Thus, intracellular signals that are induced by paclitaxel may play significant roles in mediating the clinical efficacy of this drug and are worthy of further in-depth analysis. The involvement of JNK in paclitaxel-mediated cell death suggests that artificially enhancing JNK activation may be of value in enhancing the therapeutic efficacy of paclitaxel or in treating non-paclitaxel-responsive tumors.

[5] L.-F. Lee and J. P.-Y. Ting, unpublished data.

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