Microtubule Dysfunction Induced by Paclitaxel Initiates Apoptosis through Both c-Jun N-terminal Kinase (JNK)-dependent and -Independent Pathways in Ovarian Cancer Cells*

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The antineoplastic agent paclitaxel (Taxol™), a microtubule stabilizing agent, is known to arrest cells at the G2/M phase of the cell cycle and induce apoptosis. We and others have recently demonstrated that paclitaxel also activates the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) signal transduction pathway in various human cell types, however, no clear role has been established for JNK/SAPK in paclitaxel-induced apoptosis. To further examine the role of JNK/SAPK signaling cascades in apoptosis resulting from microtubular dysfunction induced by paclitaxel, we have coexpressed dominant negative (dn) mutants of signaling proteins of the JNK/SAPK pathway (Ras, ASK1, Rac, JNKK, and JNK) in human ovarian cancer cells with a selectable marker to analyze the apoptotic characteristics of cells expressing dn vectors following exposure to paclitaxel. Expression of these dn signaling proteins had no effect on Bcl-2 phosphorylation, yet inhibited apoptotic changes induced by paclitaxel up to 16 h after treatment. Coexpression of these dn signaling proteins had no protective effect after 48 h of paclitaxel treatment. Our data indicate that: (i) activated JNK/SAPK acts upstream of membrane changes and caspase-3 activation in paclitaxel-initiated apoptotic pathways, independently of cell cycle stage, (ii) activated JNK/SAPK is not responsible for paclitaxel-induced phosphorylation of Bcl-2, and (iii) apoptosis resulting from microtubule damage may comprise multiple mechanisms, including a JNK/SAPK-dependent early phase and a JNK/SAPK-independent late phase.

Paclitaxel (Taxol™) is an antineoplastic agent specifically targeting microtubules (1, 2) and extensive studies indicate that paclitaxel arrests cells at the G2/M phase of the cell cycle (3). While mitotic arrest of paclitaxel-treated cells has been observed to initiate apoptosis (4–6), the biochemical events downstream of kinetic stabilization of microtubule dynamics which lead to apoptosis remain largely unclear (3). Furthermore, substantial evidence indicates that the G2/M arrest of the cell cycle may not be the only mechanism to induce apoptosis (7–10); additional phosphoregulatory pathways may be involved in inducing apoptosis (11–13).

We and others have recently demonstrated that paclitaxel activates the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathways in a variety of human cells through microtubular interactions (14, 15). The JNK/SAPK signaling pathways respond to various stress-related stimuli and are involved in initiation of apoptosis in many cell types (16–20). Whether JNK/SAPK activation is required for paclitaxel-induced apoptosis has remained unclear, however, it is known that paclitaxel induces phosphorylation of Bcl-2 (11, 12) and that Bcl-2 can be phosphorylated by activated JNK/SAPK (21).

The purpose of this study was to examine whether inhibition of the JNK/SAPK signaling pathway protects cells from paclitaxel-induced apoptosis and/or abrogates paclitaxel-induced phosphorylation of Bcl-2. Our results demonstrate that expression of dn-ASK1 (apoptosis signal-regulating kinase 1), dn-Rac, dn-JNKK, or dn-JNK, while exerting no effects on phosphorylation of Bcl-2, inhibits apoptosis induced by paclitaxel treatment up to 16 h. The present study clearly indicates that activation of the JNK/SAPK signaling cascade promotes early phases of paclitaxel-induced apoptosis, independently of cell cycle stage or Bcl-2 phosphorylation.


d---Independent Pathways in Ovarian Cancer Cells*

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†‡‡ The abbreviations used are: JNK, c-Jun N-terminal kinase; 7-AAD, 7-amino actinomycin D; ASK1, apoptosis signal-regulating kinase 1; Bcl-2, oncoprotein identified in B-cell leukemia/lymphoma-2; Cdk, cyclin-dependent kinase; CMV, cytomegalovirus; dn, dominant-negative; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin epitope of influenza virus; JNKK, JNK kinase; PARP, poly(A)DP-ribose polymerase; PE, phycocerythrin; PE-GFP, plasmid enhanced green fluorescent protein; PKA, protein kinase A; SAPK, stress-activated protein kinase; wt, wild type; GST, glutathione S-transferase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Me2SO, dimethyl sulfoxide.
JNK/SAPK Promotes Paclitaxel-induced Apoptosis

We used the colorimetric substrate Ac-DEVD-p-nitroanilide (Calbiochem) for caspase-3 assays in a procedure modified from the manufacturer’s protocol. Briefly, aliquots of sonicated cell lysate were incubated in lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM dithiothreitol, 0.1 mM EDTA), incubated with 200 μM Ac-DEVD-p-nitroanilide in assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol) in 96-well plates at 37 °C for 24 h. Absorbance of the cleaved product was measured at 405 nm in a BioKinetic EL340 microplate reader (Bio-Tek Instruments, Winooski, VT).

DNA Fragmentation Assays—Identification of the ladder pattern of DNA fragmentation in 1.6% agarose gel was previously reported (32). To quantify fragmented DNA in apoptotic cells, transfected cells were co-transfected with pEGFP and wt or dn expression vectors, 10⁵ trypsinized cells were incubated at room temperature for 15 min with 5 μl of phycoerythrin (PE)-conjugated annexin-V (PharMingen, San Diego, CA) and 0.125 μg/ml of 7-amino actinomycin D (7-AAD) (Sigma) in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) and analyzed by flow cytometry with a FACStarPlus (Becton Dickinson, San Jose, CA). Fluorochromes such as green fluorescent protein expressed by pEGFP, PE-annexin V, and 7-AAD were excited by laser tuned to 488 nm and emissions were detected at 507, 575, and 650 nm, respectively. Data of 10,000 cells from each sample were analyzed with the CellQuest software (Becton Dickinson, San Jose, CA). To compare other apoptotic characteristics in cells with or without expression of dn vectors, the green fluorescent protein positive and negative cells were sorted using a FACStarPlus flow cytometer and analyzed separately.

Western Blot Analyses—Aliquots of cell lysate containing equal protein mass were resolved on SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with anti-HA epitope (Boehringer-Mannheim), anti-Myc (Calbiochem, San Diego, CA), anti-ASK1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PARP (Upstate Biotechnology, Lake Placid, NY), anti-Bcl-2 (DAKO, Carpinteria, CA) antibodies followed by relevant second antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology). After washing, proteins were detected by chemiluminescence (ECL, Amersham) as described previously (14). A CMV promoter-driven enhanced green fluorescent protein construct, pEGFP (CLONTECH, Palo Alto, CA), was co-transfected as a selectable marker for transfected cells. Stock solutions of paclitaxel, actinomycin-D, and cisplatin (all from Sigma) were prepared in DMSO at concentrations of 10, 1, and 50 μM, respectively. In this study, 1 μM paclitaxel was used to treat cultured cells.

An Annexin V Binding, Flow Cytometric Analyses, and Sorting— Twenty-four h after co-transfection of BR cells with pEGFP and wt or dn expression vectors, 10⁵ trypsinized cells were incubated at room temperature for 15 min with 5 μl of phycoerythrin (PE)-conjugated annexin-V (PharMingen, San Diego, CA) and 0.125 μg/ml of 7-amino actinomycin D (7-AAD) (Sigma) in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) and analyzed by flow cytometry with a FACStarPlus (Becton Dickinson, San Jose, CA). Fluorochromes such as green fluorescent protein expressed by pEGFP, PE-annexin V, and 7-AAD were excited by laser tuned to 488 nm and emissions were detected at 507, 575, and 650 nm, respectively. Data of 10,000 cells from each sample were analyzed with the CellQuest software (Becton Dickinson, San Jose, CA). To compare other apoptotic characteristics in cells with or without expression of dn vectors, the green fluorescent protein positive and negative cells were sorted using a FACStarPlus flow cytometer and analyzed separately.

RESULTS

Expression of dn-ASK1, dn-Rac, dn-JNKK, or dn-JNK Slightly Alleviates Cytotoxicity Induced by Paclitaxel Treatment for 16 h—To determine whether inhibition of the JNK/SAPK signaling cascade abolishes apoptosis in cells treated with paclitaxel, we analyzed paclitaxel-induced apoptosis among ovarian cancer BR cells transfected with dn-Ras, dn-ASK1, dn-Rac, dn-JNKK, or dn-JNK, along with pEGFP to allow selection of transfected cells. The efficacies of these dn expression vectors for inhibition of the JNK/SAPK have been demonstrated previously (14). Following paclitaxel treatment for the indicated times, transfected cells were stained with both PE-conjugated annexin-V (binding to both apoptotic cells and dead cells) and the viability dye 7-AAD (staining dead cells but not early apoptotic cells), and analyzed by flow cytometry. A, early apoptotic cells (annexin-V positive/7-AAD negative) in LR region were differentiated from dead cells (7-AAD positive) in UR region. The UL and LL regions contain dead cells (7-AAD positive) and live cells (annexin-V negative/7-AAD negative), respectively. B and C, to analyze early apoptotic cells among transfected cells, we first excluded dead cells (7-AAD positive) from the whole population shown in B and only analyzed live cells by profiles of EGFP and PE-conjugated annexin-V. Percent of early apoptotic cells among transfected (green) cells were calculated by: 100% × UR/(UR + LR) shown in C.

Percentages of early apoptotic cells among transfected cells treated with paclitaxel for 16 or 48 h are summarized in Table I. Data presented as the (percent in paclitaxel-treated cells - percent in vehicle-treated cells) to differentiate early apoptotic cells (annexin-V positive/7-AAD negative) from dead cells (7-AAD positive) (Fig. 1).
percent in Me\textsubscript{2}SO-treated cells) ratios among cells expressing different dn signaling proteins are compared in Fig. 2. In transfected cells without paclitaxel treatment, expression of dn vectors decreased the percentage of early apoptotic cells. These decreases in basal levels of apoptosis were specific for expression of these dn expression vectors because expression of irrelevant genes in pCMV-lacZ, pCDNA3 kinase dead-p70S6kinase, or expression of these dn expression vectors because expression of irrelevant genes in pCMV-lacZ, pCDNA3 kinase dead-p70S6kinase, or pCMV-CD20 did not decrease basal apoptosis (data not shown). Treatment with paclitaxel for 16 h significantly increased apoptosis in both control vector-transfected and dn-Ras-transfected cells (p < 0.05), but the induction of apoptosis by paclitaxel was decreased to statistically insignificant levels (NS) in cells expressing dn-ASK1, dn-Rac, dn-JNKK, or dn-JNK (Table I and Fig. 2A). In contrast to 16 h treatment with paclitaxel, 48 h of treatment significantly (p < 0.05) increased early apoptotic cells in all transfected cells irrespective of expression of dn vector types (Table I and Fig. 2B).

To verify that expression of dn signaling proteins efficiently inhibited endogenous JNK/SAPK activity, we co-transfected BR cells with pEGFP and dn expression vectors, isolated transfected cells by flow cytometry using expression of pEGFP (green fluorescence) as a selectable marker, treated with paclitaxel, and analyzed by immunocomplex JNK assay. We isolated only live (7-AAD negative) green cells expressing transfected vectors (Fig. 3A) and verified expression of dn signaling proteins by Western blot analyses (Fig. 3, B and C).

The inability of dn vectors to protect cells from apoptosis following a 48-h treatment with paclitaxel did not result from decreased expression of dn signaling proteins. We have confirmed substantial expression of EGFP by fluorescent microscopy (data not shown) and expression of dn signaling proteins by Western blot analyses up to 48 h of paclitaxel treatment (Fig. 3, B and C). At 48 h of treatment, protein levels of dn-JNK or dn-ASK1 in Me\textsubscript{2}SO-treated cells remained as high as those at earlier time points; whereas those in paclitaxel-treated cells slightly decreased, perhaps resulting from a general protein degradation during apoptosis (Fig. 3, B and C). Despite its slight decrease in later time points of paclitaxel treatment, expression of these dn signaling proteins efficiently suppressed paclitaxel-induced JNK activation through all the time points (Fig. 3, B and C).

Collectively, these results suggest that expression of dn-ASK1, dn-Rac, dn-JNKK, or dn-JNK transiently protects ovarian cancer cells from paclitaxel-induced apoptosis up to 16 h. When treated with paclitaxel for a longer time, such as 48 h, cells may undergo apoptosis through additional, JNK/SAPK-independent mechanisms.

### Expression of dn Signaling Proteins of the JNK/SAPK Pathway Does Not Alter Cell Cycle Profiles of Paclitaxel-treated Cells—To investigate whether these dn signaling proteins inhibited paclitaxel-induced apoptosis through regulation on cell cycle progression, we analyzed cell cycle profiles of cells expressing dn signaling proteins at 16, 24, and 48 h of paclitaxel treatment. Compared with cells expressing control vector, expression of dn-ASK1, dn-Rac, dn-JNKK, or dn-JNK did not change cell cycle profiles, nor interfere with mitotic arrest of cells after paclitaxel treatment (Fig. 4).

### Apoptotic Characteristics in Paclitaxel-treated Cells Are Abrogated by Blockage of the JNK/SAPK Pathways—To examine the time course of inhibition of apoptosis by dn signaling proteins, we treated isolated cells expressing control vector or dn signaling proteins with paclitaxel and quantified DNA fragmentation with ELISA. Expression of dn-ASK1, dn-Rac, dn-JNKK, or dn-JNK significantly inhibited paclitaxel-induced DNA fragmentation up to 16 h, whereas the inhibition declined at 24 h and completely disappeared at 36 h of treatment (Fig. 5A). In transfected cells treated with paclitaxel for 16 h, expression of dn-ASK1, dn-Rac, dn-JNKK, or dn-JNK significantly inhibited caspase-3 activation by paclitaxel (Fig. 5B) and suppressed paclitaxel-induced PARP cleavage (Fig. 5C). These results are consistent with data given above for annexin-V binding (Table I and Fig. 2), again indicating that inhibition of the JNK/SAPK signaling pathways transiently protects ovarian cancer cells from paclitaxel-induced apoptosis.

### Overexpression of wt-ASK1, wt-Rac, or wt-JNK Promotes Apoptosis—Our data would predict that overexpression of wt signaling proteins of the JNK/SAPK pathways may promote paclitaxel-induced apoptosis. Flow cytometric analyses on annexin-V binding confirmed that overexpression of wt-ASK1, wt-Rac, of wt-JNK significantly (p < 0.05) promoted apoptosis in both Me\textsubscript{2}SO-treated and 16 h paclitaxel-treated BR cells (Fig. 6A). Consistent with flow cytometric data, overexpression of wt-ASK1, wt-Rac, or wt-JNK also significantly (p < 0.05) increased basal and paclitaxel-induced DNA fragmentation (Fig. 6B) and the increases in apoptosis measured by two independent methods are very similar. Western blot analyses with an anti-HA epitope antibody (12CA5) and an anti-Myc epitope antibody (9E10) confirmed expression of HA-wt-ASK1,
HA-wt-JNK, and Myc-wt-Rac (Fig. 6C, upper panel) and immunocomplex kinase assays verified the augmentation of paclitaxel-induced JNK activation by overexpressed signaling proteins (Fig. 6C, lower panel). The increases in the apoptosis induced by paclitaxel above those due to overexpression of wt signaling proteins were the same as that in paclitaxel-treated, control-transfected cells (Fig. 6, A and B). Therefore, the effects of paclitaxel and overexpression of wt signaling proteins appear to be additive. However, among cells overexpressing these wt signaling proteins, the 8–12-fold JNK activation induced by paclitaxel (Fig. 6C) was not proportional to the 2.5–3.5-fold increase of apoptosis (Fig. 6, A and B), suggesting that activation of JNK is not linearly related to apoptosis.

Suppression of JNK/SAPK Activation Specifically Inhibits Apoptosis Induced by 16-h Paclitaxel Treatment But Not That Induced by Actinomycin D or Cisplatin—To examine whether the JNK/SAPK signaling cascade plays a specific role in apo-
ptosis resulting from microtubular dysfunction, we compared JNK activations and cytotoxicities in ovarian cancer cells treated with paclitaxel or treated with two DNA targeting agents, actinomycin D and cisplatin. Treatment with 5 μM actinomycin D or 50 μM cisplatin for 16 h induced a comparable ladder pattern of DNA fragmentation with that induced by paclitaxel treatment (Fig. 7A), whereas only paclitaxel significantly activated JNK (Fig. 7B). Moreover, expression of dn-ASK1 or dn-JNK only inhibited DNA fragmentation induced by 16-h paclitaxel treatment but not that induced by actinomycin D or cisplatin (Fig. 7C).

**DISCUSSION**

Activation of the JNK/SAPK signaling pathways has been mechanistically implicated in regulation of apoptosis (16–20), however, the roles of JNK/SAPK in promoting (33–35) or preventing apoptosis (36, 37) differ, depending on both cell type and apoptosis-triggering stimuli (17, 19). Furthermore, in addition to apoptosis, JNK/SAPK activation may be involved in proliferation (38, 39) and oncogenic transformation (40). On the
other hand, apoptosis itself can be considered as a form of stress, hence, JNK/SAPK activation may be a stress response secondary to apoptosis, rather than a primary mediator in apoptotic pathways (19). We have previously demonstrated that in BR ovarian cancer cells treated with paclitaxel, activation of JNK/SAPK reaches a peak at 2 h when apoptosis is still minimal (14), suggesting that JNK/SAPK activation is not a secondary response to paclitaxel-induced apoptosis.

One of the early changes in apoptotic cell membranes is externalization of phosphatidylserine, which exerts high affinity to annexin-V. Therefore, increased binding of annexin-V is a sensitive indicator for apoptosis (41). Apoptotic cells are also characterized by increased activities of caspases, cleavage of 112-kDa poly(ADP-ribose) polymerase (PARP) into a 86-kDa species, and DNA fragmentation (42, 43). We have previously demonstrated that paclitaxel induces characteristic apoptotic

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**FIG. 6.** Overexpression of wt-ASK1, wt-Rac, or wt-JNK1 promotes apoptosis. A, BR cells were co-transfected with pEGFP and expression vectors for control, wt-ASK1, wt-Rac, or wt-JNK1 for 24 h, treated with MeSO (DMSO) (solid bars) or 1 μM paclitaxel (hatched bars) for 16 h, stained with PE-conjugated annexin-V and 7-AAD, and analyzed by flow cytometry as described in the legend to Fig. 1. B, BR cells overexpressing wt-ASK1, wt-Rac, or wt-JNK1 were isolated by flow cytometric sorting, treated with MeSO (solid bars) or 1 μM paclitaxel (hatched bars) for 16 h, and DNA fragmentation was quantified by ELISA. OD405 readings of control-transfected cells treated with MeSO are set as 100 arbitrary units. Data shown are mean ± S.E. (n = eight or four) from several independent experiments. C, isolated BR cells expressing control vector, HA-wt-ASK1, HA-wt-JNK1, or Myc-wt-Rac were treated with MeSO or 1 μM paclitaxel (PTX) for 3 h and expression of theses epitope-tagged, wt signaling proteins were confirmed by Western blot analyses with an anti-HA (12CA5) antibody or an anti-Myc (9E10) antibody. Endogenous JNK activities in these lysates were measured by immunocomplex kinase assay using an anti-JNK antibody for immunoprecipitation and GST-c-Jun as substrate. Non-specific bands also recognized by the 12CA5 antibody are labeled by asterisks (*).

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**FIG. 7.** Expression of dn-ASK1 or dn-JNK specifically inhibits DNA fragmentation induced by paclitaxel but not that induced by actinomycin-D or cisplatin. A, treatment with 1 μM paclitaxel (PTX), 5 μM actinomycin D (ACTD), or 50 μM cisplatin (CDDP) for 16 h induced the ladder pattern of DNA fragmentation resolved in 1.6% agarose gel. B, BR cells were treated with paclitaxel, actinomycin D, or cisplatin for 8 or 16 h, and JNK activities and JNK protein levels were measured by immunocomplex kinase assay and Western blot analysis, respectively. Relative intensities of bands are shown as fold (shown in parentheses) of MeSO-treated control. C, 24 h after transfection, sorted cells expressing control vector, dn-ASK1, or dn-JNK were treated with paclitaxel (solid bars), actinomycin D (hatched bars), or cisplatin (checked bars) for 16 h. DNA fragmentation were quantified by ELISA. Data shown are mean ± S.E. (n = 3) from a representative experiment.
morphology (23, 44) and DNA fragmentation assayed by both gel electrophoresis and the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) method in ovarian cancer cells (44). By analyzing these biochemical apoptotic characteristics in paclitaxel-treated cells overexpressing wt or dn signaling proteins of the JNK/SAPK pathways, our results herein demonstrate an apoptosis-promoting role for JNK/SAPK in ovarian cancer cells treated with paclitaxel (Figs. 2, 5, and 6). Since suppression of the JNK/SAPK signaling cascade only protects cells from paclitaxel-induced apoptosis but not from apoptosis induced by DNA targeting agents, actinomycin-D or cisplatin (Fig. 7), the present study further suggests an apoptosis-promoting role of the JNK/SAPK cascade specifically in apoptotic process resulting from microtubular dysfunction.

JNK/SAPK has been shown to be involved in activation of caspases that are required for execution of the apoptotic process (34, 46–48). The role of JNK/SAPK in activation of caspases is, however, not straightforward. JNK/SAPK could be either upstream (34, 45) or downstream (46–48) of caspase activation, depending on cell type and apoptosis-initiating agents. Since paclitaxel has been shown to activate caspases (49, 50), we analyzed the role of JNK/SAPK in caspase-3 activation of paclitaxel-treated cells. Our data show that inhibition of the JNK/SAPK cascade prevented paclitaxel-induced caspase-3 activation, PARP cleavage, and DNA fragmentation (Fig. 5), indicating that JNK/SAPK is upstream of caspase-3 activation in paclitaxel-initiated apoptosis. These and other studies, where anticancer agents were used to induce apoptosis (34, 45), suggest that JNK/SAPK acts upstream of caspase activation in chemotherapy-initiated apoptosis.

Intriguingly, while expression of dn-Ras decreased basal levels of apoptosis (Table I), it did not significantly abrogate paclitaxel-induced apoptosis (Table I and Fig. 2). Although we previously reported that both Ras and ASK1 are required for optimal activation of JNK/SAPK by paclitaxel (14), results of the present study failed to clarify a pro- or anti-apoptosis role for Ras in the apoptotic process initiated by paclitaxel. In addition to activation of mitogen-activated protein kinase and JNK/SAPK pathways, Ras may also provide a survival signal that is mediated by the phosphoinositide 3’-OH kinase dependent activation of the protein kinase B/Akt (51). Furthermore, inhibition of Ras activity induces apoptosis (52) and exerts synergistic inhibition of cell growth with paclitaxel (53). These studies further collaborate the complex and multifunctional roles of Ras in both cell growth and apoptosis.

In contrast to Ras, it is clear that the related small G-protein, Rac, has a critical role in paclitaxel-induced apoptosis at 16 h, probably via activation of JNK/SAPK. Previous studies have demonstrated that Rac activate the JNK/SAPK pathway (54–58) and our studies clearly indicate that microtubule damage activates Rac (Fig. 6C) and that Rac is at least partially responsible for JNK activation and the resulted apoptosis (Figs. 2, 5, and 6). It is interesting that among the upstream signaling proteins employed in this study, the wt-ASK1 appeared to have the highest apoptosis-promoting effect (Fig. 6). Possible explanations for these observations include that ASK1, when overexpressed, may also activate other apoptosis-related kinase such as p38 (27–30).

Since expression of dn signaling proteins remained largely intact at 48 h after Me2SO treatment (Fig. 3), the disappearance of protection from basal apoptosis cannot be explained by a decrease of dn signaling proteins. Forty-eight hours of treatment with Me2SO (vehicle control) at a 0.1% final concentration did not activate JNK/SAPK (14) or initiate apoptosis. However, liposome-mediated transfection with control DNA into BR cells was observed to exert mild cytotoxicity and caspase-3 activation,2 hence it is possible that transfection by itself may initiate an apoptotic process that eventually overrides the transient protection by dn signaling proteins of the JNK/SAPK pathway. Nevertheless, treatment with paclitaxel for 48 h caused a further significant increase in apoptosis above the relatively high basal levels (Fig. 2B and 5A, Table I), suggesting coexistence of multiple apoptotic pathways. This suggestion is supported by the ability of dn signaling proteins to inhibit apoptosis in both control and paclitaxel-treated cells at 16 h but not 48 h treatment. Therefore, the pathways leading to apoptosis after the 16-h versus 48-h treatment appear to be fundamentally different.

Multiple mechanisms have been suggested to be involved in paclitaxel-induced apoptosis, such as: abortive mitosis after paclitaxel-induced G2/M block (4–6), activation of p34cdc2 (50, 59, 60) and other Cdk’s (61, 62), activation and local release of an apoptosis-inducing cytokine (9), and induction of transcription regulators and enzymes that modulate apoptosis (10). Whereas overexpression of wt-ASK1, wt-Rac, or wt-JNK promotes apoptosis in BR cells (Fig. 6), expression of dn-ASK1, dn-Rac, dn-JNKK, or dn-JNK only transiently inhibits paclitaxel-induced apoptosis (Table I and Fig. 2). Protection from paclitaxel-induced DNA fragmentation by expression of these dn signaling proteins disappeared when cells were treated for 24–48 h (Fig. 5A). These results are in agreement with our previous report that paclitaxel induces JNK/SAPK activation in BR cells is transient with a peak at 2–4 h and declines afterward (14). Since suppression of the JNK/SAPK signaling cascade does not alter cell cycle profiles nor interfere with the paclitaxel-induced mitotic arrest that peaks at 24 h of paclitaxel treatment (Fig. 4), G2/M block of the cell cycle may mainly

2 T-H. Wang, unpublished data.
account for the later phase of paclitaxel-induced apoptosis. Our results further suggest that the late phase of paclitaxel-induced apoptosis is independent of JNK/SAPK activity. Several recent studies have suggested that catastrophic activity of Cdk5 may be a terminal effector in apoptotic pathway (63, 64).

Results in this study do not support the hypothesis that activated JNK/SAPK is required for phosphorylation of Bcl-2 in paclitaxel-treated cells (21). Bcl-2 is known to protect cells from apoptosis (65) and paclitaxel has been shown to induce both phosphorylation of Bcl-2 and apoptosis (11, 12, 66, 67). However, the roles of Bcl-2 phosphorylation in promoting (11, 12, 66, 67) or inhibiting apoptosis (70, 71) remain controversial.

Some reports suggest that Bcl-2 may act upstream of JNK/SAPK activation peaked at 2 h (14), while Bcl-2 phosphorylation and the G1/M arrest of the cell cycle required 12–16 h of paclitaxel treatment. These temporal differences suggest that phosphorylated Bcl-2 is unlikely to act upstream of JNK/SAPK. The observations that inhibition of JNK/SAPK did not interfere with paclitaxel-induced G1/M arrest and Bcl-2 phosphorylation (Figs. 4 and 8) suggest that paclitaxel-activated JNK/SAPK is independent of Bcl-2 phosphorylation occurring in paclitaxel-treated cells. Recent reports further demonstrate that phosphorylation of Bcl-2 occurs only in cells blocked at G1/M phase after paclitaxel treatment (66, 74). Therefore, JNK/SAPK activation and Bcl-2 phosphorylation may reside in distinct, independent pathways. Instead of the JNK/SAPK cascade, PKA activation and Bcl-2 phosphorylation may reside in distinct, independent pathways. The role(s) of Bcl-2 phosphorylation in promoting (11, 12, 66, 67) or inhibiting apoptosis (70, 71) remain controversial.

Our results for the first time, identify a Bcl-2 phosphorylation-independent role of JNK/SAPK in promoting paclitaxel-induced apoptosis and demonstrate that multiple mechanisms are involved in apoptosis resulting from microtubule damage, including a JNK/SAPK-dependent early phase and a JNK/ SAPK-independent late phase.

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