c-Jun N-terminal Kinase Contributes to Apoptotic Synergy Induced by Tumor Necrosis Factor-related Apoptosis-inducing Ligand plus DNA Damage in Chemoresistant, p53 Inactive Mesothelioma Cells* 

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Apoptotic resistance of cancer cells may be overcome by the combination of treatments that activate the two major apoptotic pathways: (i) the death receptor pathway activated by death ligands and (ii) the DNA damage pathway activated by chemotherapy. We have previously shown that mesothelioma cells, resistant to most treatments, are sensitive to the combination of the death ligand tumor necrosis factor-related apoptosis inducing ligand (TRAIL/Apo2L) plus chemotherapy. We investigated a possible role for c-Jun N-terminal kinase (JNK) in the synergistic effect, knowing that JNK can be activated separately by TRAIL and by DNA damage. We chose to study the M28 and REN human mesothelioma cell lines, which are p53-inactivated, to avoid an interaction between p53 and JNK. We showed that JNK was activated by TRAIL and by etoposide and that the activation was enhanced by the combination of the two treatments. We found this activation to be caspase-independent. To inhibit the JNK pathway, we used either dominant-negative constructs of JNK1 and JNK2 (compared with dominant-negative caspase 9) or a chemical inhibitor of the JNK pathway (SP600125). In cells treated with TRAIL plus etoposide, JNK inhibition increased cell survival and decreased apoptosis significantly. In transfected M28 cells, the effect of JNK inhibition was as great as that of the dominant-negative caspase 9 construct. We conclude that JNK contributes to the synergistic effect of TRAIL combined with DNA damage by mediating signals independent of p53 leading to apoptosis.

Defects in normal apoptotic programs contribute to the formation of tumors (1) and interfere with the tumor response to conventional therapy (2, 3). Abnormal apoptotic responses may arise from defective apoptotic machinery, as by mutation in p53 or up-regulation of anti-apoptotic Bcl-2 (B-cell lymphoma-2), which provide both a survival advantage and resistance to therapy (4). Among strategies to bypass sites of apoptotic resistance are the use of death receptor ligands that can directly engage their receptors and thereby recruit and activate an initiator caspase, caspase 8. DNA damaging agents engage apoptosis in a parallel fashion by altering mitochondrial function, thereby releasing pro-apoptotic molecules such as cytochrome c from the intermembranous space, resulting in part in an activation of another initiator caspase, caspase 9. These initiator caspases can then activate downstream caspases such as caspase 3 leading to a cleavage of key cellular components as the terminal and irreversible phase of apoptotic death. Simultaneous activation of the two alternate pathways, also referred to as the extrinsic (death receptor) and intrinsic (damage) pathways, may induce common signaling pathways that mediate cross-talk and amplification of the resultant apoptosis.

One potential apoptotic signaling pathway known to be engaged by both death receptors and by DNA damage is the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway (9). Although the pathway is strongly activated by a variety of apoptotic stimuli, the role of JNK in apoptosis is unclear, because it has been shown to be pro-apoptotic, neutral, or anti-apoptotic in different settings (9, 10). In many circumstances, single agents, such as chemotherapeutics alone or death receptor ligands alone, induce JNK signaling but do not depend on JNK signals for apoptotic responses. For example, a lack of dependence on JNK signals has been shown for apoptosis because of chemotherapeutic agents such as etoposide (11) or doxorubicin (12) or to death receptor activation via TRAIL (13, 14). However, when different stimuli given simultaneously produce amplified responses, the amplification of these two pathways may involve cross-talk utilizing such stress signals. Indeed, in the case of proliferative stimuli, JNK signaling may be involved in synergistic responses (15). In the

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† The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MKK, mitogen-activated protein kinase kinase; X-gal, 5-bromo-4-chloro-3-indolyl-D-galactopyranoside; PBS, phosphate-buffered saline.
case of apoptotic stimuli, induction of JNK signaling has been proposed to enhance apoptotic responses to tumor necrosis factor (16) and to Fas (17, 18). JNK signals could thus be important for synergistic apoptotic responses and, if so, could potentially be manipulated to potentiate cancer therapies. We have previously described synergistic apoptotic responses in chemoresistant mesothelioma cell lines to the combination of TRAIL and DNA damaging agents (e.g. chemotherapy or ionizing irradiation) that were not due to up-regulation of TRAIL receptors, DR4 and DR5 (19). In part because mesothelioma is reported to express wild-type p53 (20), we investigated whether p53 was involved in either synergy or in a p53-dependent JNK activation. We found that these cells had nonfunctional p53. Therefore, we asked whether JNK signals played a p53-independent role in the synergistic apoptotic responses. We describe a significant role for JNK signals in the synergistic apoptosis induced by TRAIL and the topoisomerase inhibitor, etoposide.

EXPERIMENTAL PROCEDURES

Reagents—Human recombinant TRAIL (125 TL, histidine-tagged extracellular domain; Thr 45–Gly 66) was purchased from R & D Systems Inc. (Minneapolis, MN). The chemotherapeutic agent, etoposide, was purchased from Bedford Laboratories (Bedford, OH). Inhibitor of caspases zVAD-fmk (a pan-caspase inhibitor) was purchased from Bedford Laboratories (Bedford, OH). Inhibitor of the chemotherapeutic agent, etoposide, was purchased from Sigma. Antibodies against p53 (DO-1) and p21 were purchased from Sigma. Antibodies against total SAPK/JNK and phosphorylated mitogen-activated protein kinase signaling proteins (phospho-SAPK/JNK (Thr183/Tyr185) and phospho-c-Jun (Ser63/Ile64)) were gifts from Dr. Roger Davis (University of Massachusetts, Boston, MA) (21). Antibodies against a range of other anti-apoptosis or anti-proliferation markers were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-FLAG antibody (M2) was purchased from Sigma. Antibodies against p53 (DO-1) and p21 (F-5) and secondary antibodies were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA).

Cell Lines and Culture—The human mesothelioma line, M28, was obtained from Dr. Brenda Gerwin (NCI, National Institutes of Health, Bethesda, MD), and the human mesothelioma line REN was obtained from Dr. Steven Albeda (University of Pennsylvania, Philadelphia, PA). Primary human mesothelial cells were obtained from benign pleural effusions as described (22) under approval of the Committee on Human Research and used as controls. Tumor cell lines and primary cells were cultured in Dulbecco's modified Eagle's medium/RPMI 1640 (1:1), 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT), 1-glutamine (2 mM; Invitrogen), penicillin (100 units/ml; Invitrogen), and streptomycin (100 μg/ml; Invitrogen).

General Experimental Conditions—TRAIL and etoposide were used at concentrations found to induce a synergistic apoptosis (4–20 ng/ml TRAIL and 3–15 μg/ml etoposide). For apoptosis studies, the cells were plated at 50,000 cells/well of 12-well plates the day before and exposed to treatments for described times. Ultraviolet irradiation was used as a positive control for JNK signaling; irradiation by UV-C was performed in a Stratalinker UV cross-linker model 1800 (Stratagene, La Jolla, CA) at a dose of 40–120 J/m2.

Western Blotting Analysis—Protein analysis was performed on whole cell lysates prepared on ice from 15-cm plates of M28 cells at 70% confluence after the appropriate treatment. The lysis buffer consisted of 20 mM Tris-HCl, pH 8, 137 mM NaCl, 50 mM sodium fluoride, 10% glycerol, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate, pH 8, and protease inhibitor mixture (Calbiochem, La Jolla, CA). The protein concentrations were determined using the Bio-Rad protein assay.

The samples (40 μg/lane) were boiled for 5 min with sample buffer (0.2% w/v Tris, pH 6.8, 5% SDS, 3% glycerol, and 0.01% bromphenol blue), separated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). The membranes were blocked in 5% nonfat milk (in PBS plus 0.05% Tween 20) for 1 h at room temperature and incubated overnight at 4 °C with the primary antibody in 5% bovine serum albumin (in PBS plus 0.05% Tween 20). The membranes were washed six times for 5 min each in PBS and incubated with the secondary antibody for 1 h at room temperature in 5% nonfat milk (in PBS plus 0.05% Tween 20). After washing, the blots were developed using ECL reagent (Amersham Biosciences) and autoradiography film (Kodak). The molecular weights were determined by comparison with Bio-Rad Kaledi scope markers.

Annexin V Assay for Apoptosis—Apoptosis was detected by the binding of fluorescent protein annexin V-cy3 or annexin V-fluorescein isothiocyanate to the phosphatidylserine residues on the outer leaflet of apoptotic membranes, as previously described (19). Because fewer than 5% of cells in any condition stained with propidium iodide, necrosis was considered minimal, and all annexin-V positive cells were considered apoptotic.

The cells were trypsinized and stained according to the annexin V-cy3 or annexin V-fluorescein isothiocyanate kit (Medical & Biological Laboratories, Naka-ku Nagoya, Japan). The cells were analyzed using a FAC-Scalor flow cytometer (Becton Dickinson, Franklin Lakes, NJ), with acquisition of a total of 10,000 events/sample to ensure adequate mean data. Data analysis was performed with the CellQuest software, version 3-1 f (Becton Dickinson).

Cell Transfection—Dominant-negative caspase 9, JNK1 and JNK2 plasmids. The human mesothelioma line, M28 and REN plasmids, and empty pcDNA3 plasmid were transiently transfected in M28 cells along with a pCMV-β-galactosidase vector. The cells were plated in 6-well plates at 0.25 × 104 cells/well. The next day, a total of 1.5 μg of DNA was used at a 5:1 ratio (pcDNA:pcCMV-β-galacosidase). The cells were transfected using LipofectAMINE and LipofectAMINE PLUS reagents following the manufacturer’s instructions. At 24 h after transfection, the cells were exposed to TRAIL (4 ng/ml) plus etoposide (3 μg/ml) for 6 h. The cells were washed and fixed in 0.05% glutaraldehyde for 1 min. To identify β-galactosidase enzyme activity, the fixed cells were washed three times with PBS and stained in buffer containing X-gal (1 mg/ml X-gal in 10 mM Na2PO4, pH 7, 5 mM KF, 50 mM NaF, 5 mM KF, 50 mM NaNH4H2O, 2 mM MgCl2, 0.025% Nonidet P-40, and 0.01% SDS) overnight at 37 °C. Blue cells were counted as a ratio of total cells (blue/total) as a measure of survival in a blinded fashion and compared among the different experimental conditions. For each plasmid, the relative survival of transfected cells was calculated as the survival (blue/total cells) of cells exposed to TRAIL plus etoposide divided by the survival (blue/total cells) of cells not exposed.

Statistics—One-way analysis of variance with Tukey's posthoc test was performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA). Synergy was assessed by isobolographic analysis (23) and by showing that the apoptosis to the combination of agents was significantly greater than additive (e.g. apoptosis to TRAIL plus etoposide was significantly greater than the sum of apoptosis to TRAIL plus apoptosis to etoposide). A p < 0.05 was considered significant.

RESULTS

TRAIL plus DNA Damage Induces Synergistic Apoptosis in Mesothelioma Cells—As previously shown (19), the M28 and REN mesothelioma cell lines are not sensitive to TRAIL or etoposide when given separately but show a synergistic apoptotic response to the combination of the two treatments. At 16 h, M28 and REN cells show little apoptosis to TRAIL (20 ng/ml) or etoposide (15 μg/ml) but significantly increased apoptosis to the combination (TRAIL + etoposide) (Fig. 1). p53 Is Inactive in M28 and REN Mesothelioma Cells—To determine whether p53 could mediate this synergy, we studied the functional role of p53 in these cells. We exposed M28, REN, and primary mesothelial cells, as a positive control, to a range of UV irradiation shown to induce p53 in many cell lines (24) and harvested the cells 16 h after UV exposure for determining expression of p53. As expected, p53 protein increased in primary human mesothelial cells, but no p53 protein was detected in M28 and REN cells (data not shown).
When given alone, TRAIL (20 ng/ml) and etoposide (15 μg/ml) each induced JNK phosphorylation; when given together, these agents induced enhanced JNK phosphorylation (Fig. 3B).

**JNK Activation Precedes Apoptosis and Is Not Secondary to Caspase Activation**—In some settings, JNK signaling by TRAIL or by etoposide may be secondary to the apoptotic process itself (11, 13). To address whether activation of JNK by TRAIL plus etoposide in our studies was secondary to apoptosis, we inhibited apoptosis using the pan-caspase inhibitor zVAD-fmk. M28 cells were exposed to the combination of TRAIL and etoposide for different times (2–16 h) with or without pretreatment with zVAD-fmk (100 μM), which inhibited apoptosis. JNK phosphorylation was not inhibited by zVAD-fmk and, in fact, may have been greater after caspase inhibition, as has been described previously (Fig. 3C) (13). Thus, the JNK signals associated with synergistic apoptosis are proximal to the apoptosis itself.

**Inhibition of JNK1, JNK2, and Caspase 9 by Dominant-negative Constructs Increases Survival of M28 Cells after Exposure to TRAIL plus Etoposide**—To know whether JNK contributes to the cooperative effect of TRAIL plus chemotherapy in mesothelioma cells, we inhibited JNK by transiently transfected dominant-negative JNK1 and JNK2 plasmids and the combination of the two plasmids. We also transfected a caspase 9 dominant-negative plasmid as a control for blockade of the post-mitochondrial apoptotic pathway. Because of toxicity to REN cells, we were able only to transfect M28 successfully.

First, to confirm successful transfection, we investigated protein expression of FLAG, the peptide tag used for the pcDNA dominant-negative constructs. As a negative control, we used an empty pcDNA plasmid without a FLAG tag. FLAG expression was detected for caspase 9, JNK1, and JNK2 dominant-negative transfected cells at the expected sizes of the tagged construct; no FLAG expression was detected in the cells transfected with the empty plasmid (Fig. 4A).

Second, to confirm an inhibitory effect of dominant-negative JNK on phosphorylation of its substrate c-Jun, the cells were transiently transfected with several plasmids: pcDNA empty plasmid, dominant-negative caspase 9, JNK1 and JNK2 plasmids and the combination of the two plasmids. We also transfected a caspase 9 dominant-negative plasmid as a control for blockade of the post-mitochondrial apoptotic pathway. Because of toxicity to REN cells, we were able only to transfect M28 successfully.

Knowing that the dominant-negative constructs were expressed and inhibited c-Jun phosphorylation, we investigated the effect of the inhibition of JNK on the survival of M28 cells exposed to TRAIL plus etoposide. The cells were co-transfected with various plasmids and pCMV-β-galactosidase at a 5:1 ratio and, after 24 h, exposed to TRAIL plus etoposide for 6 h, fixed, and stained for β-galactosidase activity. The blue cells were counted as a ratio of total cells and compared among the different experimental conditions. In cells exposed to TRAIL plus etoposide, inhibition of JNK led to a significant increase of 2–3-fold in relative survival of the dominant-negative transfected cells compared with the empty plasmid transfected cells (Fig. 4C).

We confirmed this increase in survival in further experiments using JNK1 and JNK2 wild-type constructs as additional controls for transfection. Compared with transfection with wild-type plasmids, the dominant-negative plasmids increased survival by at least 2-fold in cells exposed to TRAIL plus etoposide (Fig. 4D).

**Inhibition of the JNK Pathway by Chemical Inhibitor SP600125 Decreases Apoptosis of M28 and REN Cells after...**

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**Fig. 1. Apoptosis in mesothelioma cells induced by TRAIL plus etoposide.** A, M28 cells were exposed to TRAIL (20 ng/ml), etoposide (15 μg/ml), or the combination of TRAIL and etoposide for 16 h. The percentage of apoptotic cells was determined by flow cytometry after annexin-V binding (n = 3; mean ± S.D.; *, p < 0.05 different from the sum of the apoptosis caused by TRAIL plus the apoptosis caused by etoposide). B, REN cells were exposed to TRAIL (20 ng/ml), etoposide (15 μg/ml), or the combination of TRAIL and etoposide for 16 h (n = 3; mean ± S.D.; *, p < 0.05 different from the sum of the apoptosis caused by TRAIL plus the apoptosis caused by etoposide).

**Fig. 2. p53 function in M28 and REN mesothelioma cells.** p53 protein expression increased in primary human mesothelial cells 16 h after UV irradiation but failed to show expression in M28 and REN mesothelioma cells.

**JNK Is Activated in Mesothelioma Cells by TRAIL, Etoposide, and the Combination**—To confirm that JNK can be activated in M28 and REN cell lines, we exposed the cells to UV light (40 J/m²) as a well characterized stimulus for JNK signaling and harvested them 30 min later to measure the phosphorylation of JNK and total JNK expression. We found that JNK was phosphorylated without a change in total JNK protein expression (Fig. 3A).

We then studied the effect of TRAIL, etoposide, or both. When given alone, TRAIL (20 ng/ml) and etoposide (15 μg/ml) each induced JNK phosphorylation; when given together, these agents induced enhanced JNK phosphorylation (Fig. 3B).

**JNK Activation Precedes Apoptosis and Is Not Secondary to Caspase Activation**—In some settings, JNK signaling by TRAIL or by etoposide may be secondary to the apoptotic process itself (11, 13). To address whether activation of JNK by TRAIL plus etoposide in our studies was secondary to apoptosis, we inhibited apoptosis using the pan-caspase inhibitor zVAD-fmk. M28 cells were exposed to the combination of TRAIL and etoposide for different times (2–16 h) with or without pretreatment with zVAD-fmk (100 μM), which inhibited apoptosis. JNK phosphorylation was not inhibited by zVAD-fmk and, in fact, may have been greater after caspase inhibition, as has been described previously (Fig. 3C) (13). Thus, the JNK signals associated with synergistic apoptosis are proximal to the apoptosis itself.

**Inhibition of JNK1, JNK2, and Caspase 9 by Dominant-negative Constructs Increases Survival of M28 Cells after Exposure to TRAIL plus Etoposide**—To know whether JNK contributes to the cooperative effect of TRAIL plus chemotherapy in mesothelioma cells, we inhibited JNK by transiently transfected dominant-negative JNK1 and JNK2 plasmids and the combination of the two plasmids. We also transfected a caspase 9 dominant-negative plasmid as a control for blockade of the post-mitochondrial apoptotic pathway. Because of toxicity to REN cells, we were able only to transfect M28 successfully.

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**Fig. 2. p53 function in M28 and REN mesothelioma cells.** p53 protein expression increased in primary human mesothelial cells 16 h after UV irradiation but failed to show expression in M28 and REN mesothelioma cells.
Exposure to TRAIL plus Etoposide—To confirm our results using transient transfection and to extend the study to REN cells, we used the JNK inhibitor, SP600125, to investigate its effect on the apoptotic synergistic response of TRAIL plus etoposide in M28 and REN cells (25, 26). First, to confirm the effect of the inhibitor, we treated M28 cells with or without SP600125 (20 μM) for 1 h before exposing the cells to UV (40 J/m²) and 30 min later harvested the cells for detection of phospho-c-Jun. We found that UV induction of phospho-c-Jun was inhibited by SP600125. There was no effect on total JNK protein expression (Fig. 5A).

Then M28 and REN cells were pretreated with SP600125 (10, 20, or 50 μM) or Me2SO for 1 h and exposed to TRAIL or TRAIL plus etoposide for 16 h. We observed a significant decrease in the apoptosis induced by TRAIL plus etoposide in cells treated with SP600125 (Fig. 5, B for M28 and C for REN cells). These results confirmed a role of JNK signaling in the synergistic response of TRAIL plus etoposide. There was no effect of JNK inhibition on the response to TRAIL alone.

DISCUSSION

Apoptotic synergy between death receptor pathways and DNA damage pathways holds promise for enhancing tumor responses to therapy, but the mechanism of this synergy is as yet unknown. Here we show a role for a stress-activated pathway, JNK, in mediating signals contributing to the synergistic apoptosis between TRAIL and etoposide. Although each agent alone activated the JNK pathway, no significant apoptosis resulted from this JNK signaling alone. When the two agents acted together, the resulting apoptotic response was dependent, in large part, on the JNK pathway. Furthermore, because these cell lines were found to lack functional p53, we conclude that JNK can contribute to apoptosis in the absence of functional p53, a setting relevant to most solid tumors.

Our study is unusual in that it addresses the role of JNK specifically in the setting of synergistic apoptosis. In most studies, the activation of JNK and its role in the apoptotic response of the cell has been determined in response to single stimuli. It is known, for example, that the JNK pathway is activated by TRAIL and other death ligands as well as by etoposide and other chemotherapeutic agents; in most cases, JNK signals in response to the individual stimuli are independent of the resultant apoptosis (11, 13, 14). In this study, we have confirmed that TRAIL alone or etoposide alone induces JNK phosphorylation; however, it is the combination of stimuli that induces apoptosis that can be inhibited by specific inhibition of JNK. In effect, one apoptotic stimulus appears to prime the cell to respond to the other stimulus. As such, it raises the possibility that activating JNK signals could be a means of sensitizing cells to apoptosis without the need for more toxic treatments, such as those that cause DNA damage.

The reported role of JNK in cell death continues to be complex and, at times, contradictory. The JNK pathway has been shown both to mediate survival or death in response to stress, perhaps depending on the particular cell type or on the presence of other mitogenic or environmental signals (9). Particularly in transformed or tumor cell lines, JNK signaling may

for JNK signaling. In cells harvested 30 min after exposure to UV (40 J/m²), phospho-JNK increased without a change in total JNK protein. B, JNK phosphorylation increased 2–16 h after exposure to TRAIL (10 ng/ml) or etoposide (15 μg/ml) but was enhanced after exposure to the combination of TRAIL plus etoposide. Total JNK was not different at any time. C, JNK phosphorylation was not dependent on caspases. In cells exposed to TRAIL (20 ng/ml) plus etoposide (15 μg/ml) for 2–16 h, JNK phosphorylation was not inhibited by z-VAD (100 μM), at a concentration that inhibited apoptosis, showing that JNK activation is proximal to the apoptotic process.

FIG. 3. JNK activation induced by UV irradiation, as a positive control, and by TRAIL, etoposide, and TRAIL plus etoposide in M28 and REN cells. A, JNK phosphorylation was confirmed in M28 and REN cells exposed to UV irradiation, a well characterized stimulus
function as a pro-survival pathway (27–31). In other cases, JNK signaling has been unrelated to cell fate (11, 13), possibly because JNK signals were induced by the apoptotic process itself. Finally, there is strong evidence that JNK can function as a pro-apoptotic signal (32–36). In these studies, blockade of JNK signals by antisense, dominant-negative constructs or genetic deletion has shown a role for JNK in apoptosis, whether in response to ultraviolet radiation, anticarcinogenic isothiocyanates, oxidative stress, or ceramide.

Because mesothelioma is a tumor highly resistant to therapy, strategies that would enhance its response to chemotherapy could provide a useful therapeutic adjunct (37). Mesothelioma most likely has abnormal function of the p53 pathway, e.g. because of mutation or loss of p14ARF (38) or to interaction with simian virus 40 large T antigen (39), even though the tumor often expresses wild-type p53 (20). Thus, as for most tumors, therapeutic approaches successful in p53 inactive cells would be desirable. In our p53 inactive mesothelial cells, which consistently fail to respond to single agents, the use of combination therapy with TRAIL and with DNA damaging agents (chemotherapy, γ irradiation, or ultraviolet light) has shown promise (19). To investigate a possible role for JNK in the synergy, we confirmed that JNK was phosphorylated by TRAIL and by etoposide separately, as described by others, but also showed that JNK phosphorylation was enhanced by the combination. Inhibition of caspase activity had no effect on JNK activation, demonstrating that JNK was activated proximal to apoptosis and was not caspase-dependent. Blocking the JNK pathway was then essential for determining the contribution, if any, of JNK signaling to the apoptosis.

We inhibited the JNK pathway in two main ways, by dominant-negative constructs and by a specific JNK inhibitor, SP600125 (26). In the first approach, the dominant-negative blockade of JNK had limitations: the act of transfection induced some sensitization to the cells leading to an increased apoptotic response to TRAIL alone and was not possible in the REN cells because of toxicity. Nonetheless, the dominant-negative approach showed that the inhibition of JNK1 and JNK2 significantly increased survival of transfected cells exposed to TRAIL plus etoposide. Inhibition of both JNK1 and JNK2 had no more effect than of each alone, suggesting that a maximal effect had been achieved. In addition, a maximal effect was also suggested by the similar effect with dominant-negative JNK as with a dominant-negative construct to caspase 9, the initiator 9, JNK1, or JNK2) demonstrated successful transfection of constructs. The arrowheads show FLAG detection at bands corresponding to the transfected protein. (The blot is representative of several performed on cells studied in parallel with those in C.) B, c-Jun phosphorylation was inhibited by dominant-negative JNK1 and JNK2. M28 cells were transiently transfected with empty plasmid, dominant-negative constructs (caspase 9, JNK1, or JNK2) or wild-type constructs (JNK1 or JNK2). 24 h later, the cells were exposed to UV (40 J/m²) as a standard JNK stimu- lus and harvested 30 min later for detection of P-c-Jun. Total JNK protein expression did not change. C, relative survival after TRAIL plus etoposide was higher in cells transfected with dominant-negative constructs compared with empty plasmid. M28 cells were transiently transfected with pcDNA3 (empty plasmid) or with various dominant-negative constructs (caspase 9, JNK1, JNK2, and JNK1– JNK2) at a 5:1 ratio with a β-galactosidase vector. At 24 h after transfection, the cells were exposed to TRAIL (4 ng/ml) plus etoposide (3 μg/ml) for 6 h, washed, fixed, and stained for β-galactosidase activity. The blue cells were counted as a ratio of the total. Relative survival was the survival (blue cells/total cells) of transfected cells exposed to TRAIL plus etoposide divided by the survival (blue cells/total cells) of those not exposed (n = 3; mean ± S.E.; *, p < 0.05 different from relative survival of cells with empty plasmid). D, relative survival after exposure to TRAIL plus etoposide was higher in M28 cells transfected with dominant-negative JNK1 and JNK2 compared with wild-type JNK1 and JNK2 (n = 3; mean ± S.E.; *, p < 0.05 different from relative survival of cells with wild-type plasmid). WT, wild-type; DN, dominant-negative.
was decreased by SP600125 in REN cells. REN cells pretreated for 1 h exposed to UV (40 J/m²) as a standard JNK stimulus, and harvested 30 cells was determined by flow cytometry by annexin-V binding (TRAIL or TRAIL plus etoposide for 16 h. The percentage of apoptotic mean/H11006/H9262 cells were pretreated for 1 h with SP600125 (20/H11021/H9262 min later. Total JNK expression did not differ. The confounding effect of toxicity so that the synergy of TRAIL plus etoposide would allow the cells to respond to appropriate apoptotic signals independent of the p53 pathway (46, 47). The mechanism by which JNK signals mediate apoptotic synergy is not yet clear. Such synergy could possibly result from enhanced JNK signaling, because of either an increased activity or a prolonged duration of activity, which by itself was sufficient to induce apoptosis. For example, enhanced activity of JNK may result by combining signals that separately activate the JNK kinase MKK7, such as tumor necrosis factor, and MKK4, such as stress or DNA damage (48, 49). MKK7 and MKK4 phosphorylate JNK preferentially on threonine and tyrosine, respectively, and both are required for optimal JNK activity (50). Although Fas ligand has been shown to activate MKK7 (51), the ability of TRAIL to activate MKK7 has not been reported (14). If indeed, TRAIL activated MKK7 and etoposide activated MKK4, the combination would activate JNK better than each alone and perhaps lead to apoptotic synergy. In addition, an increased duration of JNK signaling may be important for JNK-induced apoptosis (32); such an increased duration can result if one stimulus blocks an inhibition, such as by NF-κB, to the alternate stimulus (52). Apoptotic synergy could further be explained by actions distal to JNK as, for example, if JNK signals sensitized the mitochondria to respond to TRAIL-induced signals via caspase 8-mediated cleavage of the Bcl-2 homology molecule, BID. In primary fibroblasts with targeted gene disruption of JNK1 and JNK2, JNK-induced apoptosis has been shown to depend on its activity or a prolonged duration of activity, which by itself was sufficient to induce apoptosis. For example, enhanced activity of JNK may result by combining signals that separately activate the JNK kinase MKK7, such as tumor necrosis factor, and MKK4, such as stress or DNA damage (48, 49). MKK7 and MKK4 phosphorylate JNK preferentially on threonine and tyrosine, respectively, and both are required for optimal JNK activity (50). Although Fas ligand has been shown to activate MKK7 (51), the ability of TRAIL to activate MKK7 has not been reported (14). If indeed, TRAIL activated MKK7 and etoposide activated MKK4, the combination would activate JNK better than each alone and perhaps lead to apoptotic synergy. In addition, an increased duration of JNK signaling may be important for JNK-induced apoptosis (32); such an increased duration can result if one stimulus blocks an inhibition, such as by NF-κB, to the alternate stimulus (52). Apoptotic synergy could further be explained by actions distal to JNK as, for example, if JNK signals sensitized the mitochondria to respond to TRAIL-induced signals via caspase 8-mediated cleavage of the Bcl-2 homology molecule, BID. In primary fibroblasts with targeted gene disruption of JNK1 and JNK2, JNK-induced apoptosis has been shown to depend on its actions at the mitochondria (36) and to require the presence of the pro-apoptotic members of the Bcl-2 family, Bax and Bak (53). We have previously shown that mesothelial cell lines, including M28 and REN, highly express Bax and Bak (53). We have previously shown that mesothelial cell lines, including M28 and REN, highly express Bax and Bak (53). We have previously shown that mesothelial cell lines, including M28 and REN, highly express Bax and Bak (53). We have previously shown that mesothelial cell lines, including M28 and REN, highly express Bax and Bak (53). We have previously shown that mesothelial cell lines, including M28 and REN, highly express Bax and Bak (53). We have previously shown that mesothelial cell lines, including M28 and REN, highly express Bax and Bak (53). We have previously shown that mesothelial cell lines, including M28 and REN, highly express Bax and Bak (53). We have previously shown that mesothelial cell lines, including M28 and REN, highly express Bax and Bak (53). We have previously shown that mesothelial cell lines, including M28 and REN, highly express Bax and Bak (53). We have previously shown that mesothelial cell lines, including M28 and REN, highly express Bax and Bak (53).
response to therapy, especially in a system without p53. Such understanding may lead to ways to manipulate signals to enhance responses to chemotherapy or even to replace chemotherapy with equally effective and less toxic downstream signals.

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