Role of the Stress-activated/c-Jun NH₂-terminal Protein Kinase Pathway in the Cellular Response to Adriamycin and Other Chemotherapeutic Drugs*

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Mitogen-activated protein kinases (MAPKs)† are serine/threonine kinases activated by dual phosphorylation on both a tyrosine and a threonine (reviewed in Refs. 1 and 2). These enzymes are important components of signaling pathways that transduce extracellular stimuli into intracellular responses. Each MAPK cascade consists of a module of three kinases: a MAPK kinase, which phosphorylates and activates a MAPK, which in turn phosphorylates and activates a MAPK. The classical MAPK module consists of Raf kinase, MEK, and ERK and is activated in response to a variety of mitogenic signals operating through different mechanisms. Activation by receptor tyrosine kinases is the best characterized mechanism, but certain G-protein-coupled receptors and cytokine receptors are also capable of activating the MAPK cascade (2). Downstream substrates regulated by ERK include transcription factors such as Elk-1 and ATF2, protein kinases including p90rsk, and several other target proteins (1).

More recently, two other MAPK modules have been characterized. One consists of MEKK, MKK4 (or SEK1), and c-Jun NH₂-terminal kinase (JNK), which, like Raf/MEK/ERK, operate in a phosphorylation cascade (3, 4). However, unlike the classical MAPK pathway, the MEKK/SEK1/JNK module is only modestly activated by growth factors and phorbol esters and is instead strongly activated by cellular stress including heat shock, UV irradiation, protein synthesis inhibitors, and inflammatory cytokines (5). JNK is also termed stress-activated protein kinase (SAPK) (5), and two main forms (JNK1 and JNK2) have been described (6). JNK is instead strongly activated by cellular stress including heat shock, UV irradiation, protein synthesis inhibitors, and inflammatory cytokines (5). JNK is also termed stress-activated protein kinase (SAPK) (5), and two main forms (JNK1 of 46 kDa and JNK2 of 55 kDa) have been described (6). An important physiological substrate of JNK is c-Jun, and phosphorylation of two sites in the NH₂-terminal transactivation domain (Ser-63 and Ser-73) regulates transcriptional activity (6).

A third MAPK isoform is p38, a homolog of the yeast HOG1 (high-osmolality glycerol response-1) kinase (7), also termed p40 (8), reactivating kinase (9), or cytokine-suppressive anti-inflammatory binding protein (10) in independent studies. Like ERK and JNK, p38 is activated by dual phosphorylation on a tyrosine and a threonine residue, and this is catalyzed by a MEK family member, MKK3 (11). p38 is activated by inflammatory cytokines and environmental stress including osmotic shock and UV irradiation (12). An important physiological substrate of p38 is MAPK-activated protein kinase-2, which phosphorylates heat shock protein hsp27 as part of the cellular response to stress (9). Although JNK and p38 appear to reside in distinct MAPK modules, a functional overlap is likely since each can complement the hog1Δ1 yeast strain (7, 13).

Recent data suggest that JNK is also activated in response to cellular stress induced by certain DNA-damaging agents. For example, 1-β-D-arabinofuranosylcytosine (araC) (14), cis-platinum (15), and mitomycin C (15) activate JNK in NIH-3T3 fibroblasts. In this study, we sought to determine whether MAPKs play a role in the cellular stress response to other cancer chemotherapeutic drugs. We examined JNK and ERK activation in human carcinoma cells treated with Adriamycin, vinblastine, or VP-16. These agents were chosen for study
because they represent widely utilized anticancer drugs with different mechanisms of action. Adriamycin has a complex mechanism of cytotoxicity through its ability to intercalate DNA and generate superoxide; vinblastine is a microtubule inhibitor; and VP-16 is a topoisomerase II inhibitor. Since these drugs belong to the multidrug resistance (MDR) group of substrates transported by P-glycoprotein (16), JNK activity was also evaluated in MDR variant carcinoma cell lines. Our findings suggest that JNK activation is an important component of the cellular response to mechanistically diverse cancer chemotherapeutic drugs and may also play a role in MDR.

EXPERIMENTAL PROCEDURES

Materials—Polyclonal antibodies to JNK1 or ERK1 were obtained from Santa Cruz Biotechnology. Polyclonal antibodies specific for the phosphorylated forms of ERK1/2 or c-Jun were obtained from New England Biolabs Inc. GST-c-Jun(79), a fusion protein of glutathione S-transferase and residues 1–79 of human c-Jun, was either obtained from Santa Cruz Biotechnology or purified from Escherichia coli cells harboring pGEX-2T-GST-c-Jun(79) expressed in bacteria, as described by Dr. Omar Coso, Molecular Signaling Unit, National Institutes of Health. γ-[^32P]ATP (3000 Ci/mmol) was obtained from Amersham International. Tissue culture media and the SuperScript preamplification system were purchased from Life Technologies, Inc. Taq polymerase was from Fisher, and T4 polynucleotide kinase was from Promega. 12-O-Tetradecanoylphorbol-13-acetate (TPA), Adriamycin, vinblastine, 

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Preparation of Nuclear Extracts—Cell monolayers were washed in phosphate-buffered saline, collected by scraping and sedimentation, and suspended in 0.3 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 10 mM EGTA, 1 mM dithioreitol, 20 mM β-glycerophosphate, 1 mM sodium vanadate, 0.1 mM okadaic acid, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 50 μg/ml leupeptin, and 10 μM pepstatin). The cells were lysed by Dounce homogenization, and nuclei were collected by centrifugation (10 min, 1000 × g). The samples, adjusted to contain equal protein, were solubilized in SDS sample buffer with heating to 95°C for 5 min.

Immunoblot Analysis—Soluble cell lysates or nuclear suspensions (40–100 μg, as indicated in the figure legends) were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with antibodies, all of which were used at a dilution of 1:1000, except anti-phospho-ERK1/2 antibody (1:2500). Primary antibody was detected by horseradish peroxidase-conjugated secondary antibody (1:5000), which in turn was visualized using enhanced chemiluminescence (Amersham International).

RNA Extraction and cDNA PCR Analysis of MDR1 Expression—Total RNA extracted from control and MDR variant carcinoma cell populations (cultivated in six-well plates) was extracted by a small-scale procedure using RNA STAT-60 (Tel-Test “B”, Inc.). cDNA synthesis was carried out using the SuperScript preamplification system as described by the manufacturer. Amplification with primers specific for MDR1 or β2-microglobulin, the latter as an internal control, was performed as described previously (21) with certain modifications. Briefly, PCR was performed with a Perkin-Elmer GeneAmp 2400 Thermocycler, and reaction mixtures contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 200 μM each dNTP, 200 ng of each gene-specific primer, 50 ng of RNA-equivalent cDNA, and 1 unit of Taq polymerase. Sense primers were 5’-end-labeled with γ[^32P]ATP for detection of the amplified product. After an initial denaturation at 94°C for 5 min, the following step-cycle program was initiated: denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 60 s. For MDR1, 37 cycles were performed, and for β2-microglobulin, 27 cycles were performed, each followed by a final extension at 72°C for 7 min. PCR products were analyzed by SDS-PAGE (12% acrylamide) and autoradiography.

RESULTS

Activation of JNK by Adriamycin—To validate the JNK assay in KB-3 cell extracts and to examine possible effects on activity of Adriamycin treatment, lysates were prepared from cells exposed to 500 ng/ml Adriamycin for either 1 or 16 h. As controls, cells were untreated, exposed to vehicle (dimethyl sulfoxide) alone, heat-shocked, or stimulated with 100 nM TPA for 30 min. JNK activity was determined by an immunocomplex assay with GST-c-Jun(79) as substrate as described under “Experimental Procedures.” Consistent with previous reports in other cell lines (e.g. Ref. 5), JNK was strongly activated by heat shock, but was not significantly activated by TPA (Fig. 1). Significantly increased JNK activity was also observed after treatment of cells with Adriamycin for 16 h (Fig. 1). A more detailed kinetic study demonstrated that JNK activity was detectably increased after a 1-h exposure of cells to Adriamycin, with a further increase after 4 h and a stimulation of >40-fold (average of four experiments, as determined by densitometric scanning) after a 16-h exposure to the drug (Fig. 2A, upper panel). Immunoblotting of cell lysates with anti-JNK antibody revealed that JNK1 protein levels remained constant under these treatment conditions (Fig. 2A, lower panel). The concentration dependence of JNK activation by Adriamycin (16-h exposure) was next determined; enzyme activation was observed in the range of 20–500 ng/ml Adriamycin, with an increase of 40-fold at 500 ng/ml (Fig. 2B). Activation of JNK by Vinblastine and VP-16—We next ex-
examined JNK activity in KB-3 cells treated for 16 h with two other important cancer chemotherapeutic drugs, vinblastine and VP-16. Activation of JNK was observed at vinblastine concentrations of 5 ng/ml and above, with a stimulation of enzyme activity of 6.5-fold (average of three experiments) (Fig. 3A). VP-16 treatment also activated JNK, with a stimulation of enzyme activity of 4.3-fold (average of two experiments) at a concentration of 1 µg/ml (Fig. 3B). We also tested araC, which, at 10 µM for 2 h, has been reported to activate JNK in NIH-3T3 and 293 kidney cells (14). When KB-3 cells were exposed to 10 µM araC for different periods up to 40 h, we observed a modest 2-fold activation of JNK activity (data not shown).

**Effect of Drugs on c-Jun Phosphorylation**—An anticipated consequence of JNK activation is an increased phosphorylation of sites in the NH$_2$-terminal region of the nuclear substrate, c-Jun. NH$_2$-terminal c-Jun phosphorylation was monitored by gel electrophoresis of nuclear extracts and immunoblotting with an antibody specific for the phosphorylated Ser-73 form of c-Jun. As shown in Fig. 4, significantly increased phospho-Ser-73 c-Jun immunoreactivity was observed in nuclei of cells exposed to Adriamycin or vinblastine. Immunoreactivity was barely detectable in control or VP-16- or araC-treated cells under these conditions and was greater in vinblastine-treated versus Adriamycin-treated cells, despite the fact that Adriamycin was a more potent activator of JNK. In Adriamycin-treated cells, activated JNK may preferentially phosphorylate another substrate, or the time course of c-Jun phosphorylation may differ from that of JNK activation. Despite these caveats, the increased abundance of c-Jun phosphorylated on Ser-73 is consistent with JNK activation in vivo in response to Adriamycin or vinblastine treatment.

**Effect of Drugs on ERK Activation**—The results presented above indicated that certain chemotherapeutic drugs activate the SAPK pathway in KB-3 cells. To determine whether the drugs selectively activated specific MAPK cascades, ERK activation was examined in KB-3 cells treated with drugs under

![Fig. 1. Activation of JNK by heat shock or Adriamycin. JNK activity in KB-3 cell lysates was determined by immunocomplex assay as described under “Experimental Procedures.” Phosphorylated GST-c-Jun (GST-cJ) was detected by SDS-PAGE and autoradiography. Molecular mass standards (in kilodaltons) are indicated on the left. Lane 1, control (no addition); lane 2, control (dimethyl sulfoxide (DMSO) vehicle, 0.1% (v/v) final concentration); lane 3, heat shock (42°C, 35 min); lane 4, TPA (100 nM, 30 min); lanes 5 and 6, Adriamycin (ADR; 500 ng/ml, 1 or 16 h).](image1)

![Fig. 2. Time course and concentration dependence of JNK activation by Adriamycin. A, time course of JNK activation and determination of JNK protein levels. JNK activity was determined by immunocomplex assay in lysates from KB-3 cells treated with 500 ng/ml Adriamycin (ADR) for the times indicated. Upper panel, phosphorylated GST-c-Jun (79) (GST-cJ) detected by SDS-PAGE and autoradiography; lower panel, immunoblot of the respective cell lysates (40 µg of protein/lane) probed with anti-JNK antibody. The strongly immunoreactive band corresponds to JNK1. B, concentration dependence of JNK activation. KB-3 cells were treated with the indicated concentrations of Adriamycin (ADR) for 16 h. Upper panel, JNK activity; lower panel, JNK immunoblot of the corresponding cell lysates (40 µg of protein/lane). See “Experimental Procedures” for other details.](image2)

![Fig. 3. Concentration dependence of JNK activation by vinblastine or VP-16. KB-3 cells were treated with the indicated concentrations of vinblastine (VBL; A) or VP-16 (B) for 16 h. JNK activity was determined by immunocomplex assay (upper panels in A and B), and JNK protein levels by immunoblotting (lower panels in A and B) as described under “Experimental Procedures.” See the legend to Fig. 2 for other details. GST-cJ, GST-c-Jun (79).](image3)
and KB-A1 cell lines (data not shown). The MDR variants. Basal ERK activity and ERK protein level suggested that JNK is present in a more highly activated form in lines expressed similar levels of JNK protein. These results average of three independent experiments, while all three cell

was observed for the chemotherapeutic drug treatments. Some-what unexpectedly, ERK activity was increased >6-fold by heat shock, and this provided a convenient positive control for ERK activation in these cells (lane 3). TPA failed to significantly activate ERK in KB-3 cells (lane 9), but this appeared to be a cell type-specific phenomenon since TPA strongly activated ERK in K562 cells (lanes 1 and 2). In all cases, the expression level of ERK protein appeared unchanged as judged by immunoblot analysis (Fig. 5A, lower panel). To confirm these observations with an independent assessment of ERK activation, cell lysates from similarly treated cells were subjected to Western blot analysis with a phospho-ERK1/2 antibody (Fig. 5B). Phospho-ERK1/2 immunoreactivity was increased by heat shock, but was not significantly affected by the drug treatments, consistent with the ERK immunocomplex assay data. The specificity of the phospho-ERK antibody was confirmed by antibody recognition of a control protein consisting of bacterially expressed, purified ERK2 phosphorylated by MEK (Fig. 5B).

JNK Activity in Multidrug-resistant Cells—Adriamycin, vinblastine, and VP-16 share the common property of being substrates for the drug efflux pump, P-glycoprotein, which is over-expressed in many MDR cell lines (16). Since all three drugs activated JNK in KB-3 cells, it was of interest to evaluate JNK activity in the MDR derivative KB-V1 and KB-A1 cell lines, which were derived from KB-3 cells by selection for resistance to vinblastine and Adriamycin, respectively (17). Cell lysates were prepared and subjected to JNK immunocomplex assay and JNK immunoblot analysis (Fig. 6). Relative to KB-3 cells, basal JNK activity was found to be significantly increased in both MDR cell lines (4-fold for KB-V1 and 7-fold for KB-A1, average of three independent experiments), while all three cell lines expressed similar levels of JNK protein. These results suggest that JNK is present in a more highly activated form in the MDR variants. Basal ERK activity and ERK protein level were also assessed and found to be similar for the KB-3, KB-V1, and KB-A1 cell lines (data not shown).

Induction of MDR1 mRNA by Chemotherapeutic Drugs—An earlier study found that treatment with chemotherapeutic drugs of certain drug-sensitive cancer cell lines, including KB-3, K562, and H9 cells, resulted in the induction of MDR1 mRNA (21). It was suggested that MDR1 induction may be a general response to drug-induced cellular damage. We considered the possibility that JNK activation and MDR1 mRNA induction by chemotherapeutic drugs may be linked, particularly in view of the results in Fig. 6 showing increased JNK activity in MDR cells. We therefore analyzed MDR1 mRNA expression by reverse transcription-PCR in untreated and drug-treated KB-3 cells. In confirmation of the earlier findings (21), untreated cells expressed a barely detectable level of MDR1 mRNA. Treatment of cells with 200 ng/ml Adriamycin, 5 ng/ml vinblastine, 1 μg/ml VP-16, or 10 μM araC induced MDR1 expression (Fig. 7). These drug concentrations were found to be optimal for MDR1 induction in KB-3 cells. The MDR1-overexpressing KB-V1 cell line was utilized as a positive control, and β2-microglobulin mRNA levels were determined as an internal control and found to be unchanged (Fig. 7).

DISCUSSION

In this paper, we have shown that three structurally and mechanistically distinct anticancer drugs activate the stress-activated kinase, JNK, in human carcinoma cells. The most potent compound studied was Adriamycin, which maximally activated JNK 40-fold; vinblastine and VP-16 maximally acti-
activated JNK 6.5- and 4-fold, respectively. JNK activation occurred at clinically relevant drug concentrations. araC was also tested, but was a relatively poor JNK activator in this system. In all cases, increased JNK enzyme activity occurred without a change in JNK protein expression. Consequences of activated JNK include phosphorylation of pre-existing c-Jun and an increase in \( c-jun \) transcription and synthesis of new c-Jun protein (reviewed in Ref. 22). The increased abundance of c-Jun phosphorylated on Ser-73 in cells treated with Adriamycin or vinblastine provides further evidence that exposure to these agents stimulates JNK activity \( \text{in vivo} \). More detailed studies will be required to determine the temporal relationship between c-Jun phosphorylation and JNK activation in response to Adriamycin and vinblastine treatment and to determine whether VP-16 treatment influences the phosphorylation of c-Jun. The drugs examined failed to significantly activate the ERK pathway relative to the JNK pathway, thereby displaying specificity with regard to stimulation of different MAPK pathways. It remains to be determined whether other SAPKs such as p38MAPK or the newly described SAPK3 (23) are also activated by these cytotoxic drugs and whether other JNK substrates such as ATF2 are affected.

The JNK pathway is likely activated as a result of drug-induced cellular damage, but the intracellular signals linking cell damage to the stress response are incompletely defined. Many DNA-damaging drugs including Adriamycin and VP-16 induce expression of the nuclear phosphoprotein p53 (24, 25). Vinblastine, which does not directly damage DNA, has also been shown to increase the levels of transcriptionally active p53 in NIH-3T3 fibroblasts (26). Downstream events of p53 activation include cell cycle arrest at G1 and apoptosis in many cell systems (reviewed in Ref. 27). Indeed, apoptosis has been observed in several cell lines after treatment with Adriamycin (e.g. Refs. 28 and 29), VP-16 (e.g. Ref. 30), or vinblastine (e.g. Ref. 31). Consistent with these observations, overexpression of the apoptosis inhibitor Bcl-2 protects cells from the cytotoxic actions of many anticancer agents including Adriamycin, VP-16, and vinca alkaloids (reviewed in Ref. 32). Recent evidence suggests that the SAPK/JNK signaling pathway may be an important component of apoptosis and cell death induced by stressful stimuli. Zanke et al. (33) reported that stable expression of a dominant inhibiting SEK1 mutant blocked SAPK activation and conferred increased resistance to cell death induced by several different stressful stimuli including heat shock, UV irradiation, and cis-platinum. In addition, transient expression of a dominant-negative kinase-inactive SEK1 mutant reduced apoptosis induced by several JNK activators including H\(_2\)O\(_2\), UV irradiation, heat shock, or treatment with tumor necrosis factor-\( \alpha \) (34). Our finding that mechanistically distinct cytotoxic drugs activate the SAPK/JNK pathway supports the concept that JNK may play a role in the cell death pathways induced by these agents.

Although evidence has accumulated suggesting a role for the SAPK pathway in cell death, there may be other consequences of JNK activation. It is possible that, in some circumstances, SAPK/JNK activation is part of a protective mechanism to support cell repair. The degree of damage and the capacity for repair may be important determinants dictating the choice between death and survival. One well characterized mechanism of protection against cytotoxic drugs is P-glycoprotein overexpression (16). Consistent with a previous report (21), we showed that Adriamycin, vinblastine, VP-16, and araC all induced \( MDR1 \) mRNA expression in KB-3 cells (Fig. 7). With the exception of araC, which was a poor JNK activator in these cells, the concentrations of the drugs optimal for \( MDR1 \) induction were similar to those optimal for JNK activation. Although these results do not demonstrate a causal relationship between JNK activation and \( MDR1 \) induction, they do suggest a possible link between the two parameters. The fact that the MDR cell lines examined express a more highly activated form of JNK is intriguing in this regard. The presence of activated JNK could be due to activation of upstream regulators of JNK, inactivation or down-regulation of a phosphatase acting on JNK, or both in the MDR cell lines. In the context of this study, it is interesting to note that JNK activation and \( MDR1 \) expression are induced by several other common stimuli. For example, the \( MDR1 \) gene is induced by heat shock (35) and UV irradiation (36), both well established activators of JNK. The presence of a non-canonical AP-1 consensus element in the human \( MDR1 \) promoter (37) is perhaps significant since JNK enhances AP-1-dependent transcription through modulation of c-Jun phosphorylation and expression (22). Further investigation will be required to elucidate the role of JNK in the cellular response to cytotoxic drugs and the relationship of this stress-activated pathway to \( MDR1 \) expression.

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