Transient Stimulation of the c-Jun-NH$_2$-Terminal Kinase/Activator Protein 1 Pathway and Inhibition of Extracellular Signal-regulated Kinase Are Early Effects in Paclitaxel-mediated Apoptosis in Human B Lymphoblasts

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ABSTRACT

We demonstrate here that paclitaxel exposure to RPMI-1788 B lymphoblasts caused a dose- and time-dependent increase in nuclear factor activator protein 1 (AP-1) DNA binding activity. The basal DNA binding activities of nuclear factors NF-$\kappa$B and Ets were not affected by paclitaxel. Consistent with these biochemical events, paclitaxel stimulated AP-1-dependent chloramphenicol acetyltransferase (CAT) reporter gene transcription in vivo, as directed from a tetradecanoyl phorbol acetate-inducible promoter. AP-1 binding activity of nuclear extracts isolated from paclitaxel treated cells was reduced following immunodepletion with antibodies directed against individual Jun family proteins, whereas anti-cFos, anti-Fra1, and anti-FosB antibodies were not inhibitory. Paclitaxel caused a rapid and transient increase in c-Jun NH$_2$-terminal kinase (JNK) activity, a proposed mediator of stress activation pathways. By contrast, exposure to paclitaxel produced a transient reduction in the extracellular signal-regulated mitogen-activated protein kinase 2 (ERK2) activity, a proposed mediator of growth factor-stimulated proliferation pathways. Transient activation of the c-Jun-NH$_2$-terminal kinase/AP-1 pathway, together with down-regulation of ERK2 activity, may be a key event in the early response of RPMI-1788 B lymphoblasts to paclitaxel exposure.

INTRODUCTION

Paclitaxel promotes microtubule stabilization and induces cell death via apoptosis, a process characterized by cytoskeletal changes, chromatin condensation, and genomic DNA fragmentation (1–7). Recent studies suggest that paclitaxel affects the activities of several intracellular tyrosine and serine/threonine protein kinases (8–10). Croce and coworkers (11) and Blagoskionny et al. (12) found that paclitaxel induces the phosphorylation of bcl-2, an event that abrogates its antiapoptotic activity (13). Subsequent studies from these investigators found that bcl-2 is phosphorylated in response to microtubule stabilization by paclitaxel or agents that bind monomer tubulin (e.g., vincristine and vinblastine), suggesting that bcl-2 may act to serve as an intracellular sensor for microtubule integrity (14, 15).

Some of the initial studies in mammalian cells to support the notion that apoptosis is due to programmed cell death were obtained from experiments that demonstrated that de novo RNA and protein synthesis was essential for cell death (16). It was later found that RNA and protein synthesis inhibitors failed to prevent apoptosis and in some situations induced apoptosis, suggesting that cell death components pre-exist in most eukaryotic cells (17, 18). It is noteworthy that recent reports have suggested that trans-acting DNA-binding proteins, such as members of the Jun and NF-$\kappa$B/Rel families, are necessary for apoptosis in some models (19–21). Insight into the role of trans-acting proteins has been gained from studies of c-myc gene expression in immature B lymphocytes in which decreases in c-myc mRNA levels induce apoptosis (21). Transcription of the c-myc gene in WEHI-231 B cells is regulated by two $\kappa$B motifs; apoptosis-inducing agents reduce c-myc gene transcription by down-modulating NF-$\kappa$B/Rel activity (22, 23).

Signals generated in response to stimulus-induced apoptosis or cellular stress affect the activity of transcription factors via several distinct signal transduction pathways. Members of the MAP kinase family have been shown to mediate mammalian cell responses to environmental stress signals (24). For example, induction of apoptosis by $\gamma$-irradiation and tumor necrosis factor $\alpha$ results in activation of JNK (25–30); JNK phosphorylates c-Jun, Elk-1, and ATF-2, leading to their transcriptional activation (30–35). Overexpression of a dominant-interfering c-Jun mutant lacking the NH$_2$ terminus inhibits stress-induced apoptosis, suggesting that phosphorylated c-Jun may be necessary for the activation of downstream events in the cell death program (36). A recent study by Xia et al. (34) found that induction of apoptosis following nerve growth factor withdrawal leads to sustained activation of the JNK/p38 MAP kinases and reduction of ERK activity.

In this study, we sought to determine whether cells exposed to paclitaxel respond by altering the activities of environmental stress-regulated protein kinases and downstream trans-acting nuclear factors. We chose as a model for paclitaxel-induced apoptosis the human B-lymphoblastoid cell line RPMI-1788. Our results indicate that the binding activity for nuclear factor AP-1, but not NF-$\kappa$B and Ets, is induced in response to paclitaxel. The induced AP-1 is biologically functional in vivo insofar as paclitaxel stimulates transcription of a TRE-dependent CAT reporter fusion gene. We also report that these nuclear events are preceded by a rapid and transient increase in JNK activity and a down-regulation of ERK2 activity.

MATERIALS AND METHODS

Cell Culture. The human lymphoblast, RPMI-1788 (lgM-secreting) and the acute T-cell leukemia human Jurkat T (clone E6-1; Interleukin 2 secreting) cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI-1640 containing 10 mM HEPES (pH 7.5), 2 mM L-glutamine, 5 X 10$^{-5}$ M 2-mercaptoethanol, and 10% FCS (BioWhittaker, Walkersville, MD). Cells were stimulated with 100 ng/ml PMA (Sigma Chemical Company, St. Louis, MO) or growth arrested with paclitaxel (Bristol-Myers Squibb Pharmaceutical Co., Princeton, NJ). PMA and paclitaxel were dissolved in DMSO as 1 mg/ml and 25 mM stock solutions, respectively. LPS was type Salmonella typhosa 0901 from DIFCO Laboratories (Detroit, MI).

The abbreviations used are: NF-$\kappa$B, nuclear factor $\kappa$B; AP-1, activator protein 1; EMSA, electrophoretic mobility shift assay; PMA, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyltransferase; TRE, tetradecanoyl phorbol acetate-response element; MAP, mitogen-activated protein; JNK, c-Jun NH$_2$-terminal kinase; ERK, extracellular signal-regulated MAP kinase; GST, glutathione S-transferase; GSH, reduced glutathione; LPS, lipopolysaccharide; Ab, antibody.

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4 The abbreviations used are: NF-$\kappa$B, nuclear factor $\kappa$B; AP-1, activator protein 1; EMSA, electrophoretic mobility shift assay; PMA, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyltransferase; TRE, tetradecanoyl phorbol acetate-response element; MAP, mitogen-activated protein; JNK, c-Jun NH$_2$-terminal kinase; ERK, extracellular signal-regulated MAP kinase; GST, glutathione S-transferase; GSH, reduced glutathione; LPS, lipopolysaccharide; Ab, antibody.
Flow Cytometry and DNA Fragmentation. B cells (10⁶) were washed several times in PBS and then stained with 10 μg/ml propidium iodide and 40 μg/ml RNAse A for 1 h at 23°C as described (37). DNA content was measured with a FACScan II (Becton Dickinson, Inc., San Jose, CA). The incorporation of [³H]thymidine was measured by culturing in triplicate 10⁶ cells/ml in 96-well flat-bottomed microtiter plates. Approximately 6 h before the indicated times, 0.25 μCi of [³H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) was added to each well, and cells were then assayed for [³H]thymidine incorporation into chromatin with a cell harvester. Early apoptotic cells were detected by fluorescence microscopy following staining with the FITC conjugate of annexin V following the manufacturer’s instructions (CalBiochem-Novabiochem International, Cambridge, MA).

DNA fragmentation was assessed by incubating 10⁶ cells at 50°C for 18 h in 300 μl of digestion buffer [10 mM Tris (pH 8.0) 25 mM EDTA, 100 mM NaCl, 0.5% SDS, and 0.1 μg/ml proteinase K]. Cell lysates were then incubated for 2 h at 37°C with 5 μg of RNAse A and extracted as described (7). DNA was subjected to 1.2% agarose gel electrophoresis in Tris-borate EDTA buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) and stained with ethidium bromide.

EMSA. Nuclei were isolated from 10⁶ B cells and extracted in a 450 mM NaCl buffer as described by Chiles et al. (38). Binding reactions were 15-μl volumes containing 1.5 μg of nuclear extract protein, 1 μg of poly (deoxyinosinic-deoxyycytidylic acid), and 0.05 μg of [γ-³²P]ATP-labeled oligonucleotide probe. After 20 min (23°C), the reaction products were electrophoresed through a native 5% polyacrylamide/Tris-borate EDTA gel and subjected to autoradiography. The oligonucleotide probes used to detect DNA-binding proteins correspond to the human metallothionein II α gene promoter AP-1 site (TCGACGGTATCGATACGTGACTACTAATCCGCGGCG; Ref. 39), the murine junB gene promoter Ets site (GATCACGCGCTTCCTGCAAGT) (40), and a bZIP consensus site (AGTGTAGGGGAGCTTCCCAAGG; Ref. 41). Competition studies were performed by including in the binding reactions a 10-, 50-, or 100-fold excess of corresponding unlabeled of oligonucleotide for 20 min (23°C).

In some experiments, 5 μg of nuclear extract protein was incubated with 5 μg of highly specific anti-cJun, anti-JunB, anti-JunD anti-Fos, anti-Fos-B, or anti-Fra1 Abs (Santa Cruz Biotechnology, Santa Cruz, CA). After 90 min (23°C), 15 μl of a 1:1 slurry of protein G-agarose (Life Technologies, Inc., Gaithersburg, MD) were incubated with the nuclear extracts for 60 min. The immune complexes were removed by centrifugation, and the nuclear extract was assayed for AP-1 binding activity by EMSA.

DNA Transfection and Reporter Gene Expression. B cells were transiently transfected with 600 μg/ml DEAE-dextran and 10 μg of plasmid DNA (42). Cells were cultured at 3 × 10⁶ cells/ml for 24 h and treated with 60 ng/ml paclitaxel or 100 ng/ml PMA for 18 h. CAT assays were conducted as described by Chiles and Rothstein (42), the amount of acetylated and unacetylated [³H]chloramphenicol was determined by TLC, and the results of this study were performed by including in the binding reactions a 10-, 50-, or 100-fold excess of corresponding unlabeled of oligonucleotide for 20 min (23°C).

Some experiments, 5 μg of nuclear extract protein was incubated with 5 μg of highly specific anti-cJun, anti-JunB, anti-JunD anti-Fos, anti-Fos-B, or anti-Fra1 Abs (Santa Cruz Biotechnology, Santa Cruz, CA). After 90 min (23°C), 15 μl of a 1:1 slurry of protein G-agarose (Life Technologies, Inc., Gaithersburg, MD) were incubated with the nuclear extracts for 60 min. The immune complexes were removed by centrifugation, and the nuclear extract was assayed for AP-1 binding activity by EMSA.

RESULTS

Paclitaxel Inhibits DNA Synthesis and Causes Apoptosis in RPMI-1788 B Lymphoblasts. RPMI-1788 B lymphoblasts were treated with varying concentrations of paclitaxel for 48 h and then incubated with [²H]thymidine to monitor DNA synthesis (Fig. 1A). [²H]Thymidine uptake was reduced by 50% in B cells treated with 1.8 ng/ml paclitaxel, and greater than 90% [²H]thymidine uptake was blocked at concentrations exceeding 12 ng/ml. Flow cytometry of DNA content revealed that 28.5, 32.6, and 38.9% of control B lymphoblasts were distributed in G1, S and G2-M phases of the cell cycle, respectively (Fig. 1B). Paclitaxel (60 ng/ml)-treated cell populations demonstrated a 19.1% (G1), 25.3% (S phase), and 55.6% (G2-M) distribution at 24 h. Gel electrophoresis of DNA extracted from cells after paclitaxel exposure is shown in Fig. 1C. Fragmentation of chromosomal DNA was observed at 24 and 48 h following exposure to paclitaxel; DNA fragmentation was not detected at 12 h or 18 h (data not shown). Fig. 1D shows a field of control (Fig. 1D, a and b) and paclitaxel-treated (Fig. 1D, c and d) RPMI-1788 cells stained with FITC-annexin V (Fig. 1D, b and d) to detect surface phosphatidyserine, an early-stage marker for apoptosis (47). A significant population of cells exposed to paclitaxel for 24 h stained with FITC-annexin V (Fig. 1D, d). In contrast, cells cultured in medium alone exhibited little, if any, FITC-annexin V staining (Fig. 1D, b). Taken together, these results suggest that paclitaxel induces apoptosis in RPMI-1788 B lymphoblasts.

Paclitaxel Induces Nuclear Factor AP-1 Binding Activity. To study the possible regulation of nuclear targets by paclitaxel, nuclei from control and paclitaxel-treated RPMI-1788 B lymphoblasts were examined for the expression of several inducible nuclear DNA-binding factors, including AP-1 (39), Ets (40), and NF-xB (41). EMSA of nuclear extracts obtained from control cells revealed a low amount of AP-1 and Ets DNA binding activities, whereas the level of nuclear NF-xB DNA binding activity was comparatively higher (Fig. 2A). Treatment of cells with 60 ng/ml paclitaxel for 8 h led to an increase in AP-1 DNA binding activity (Fig. 2A, Lane 7). In contrast, nuclear
extract binding to either the κB- or Ets-containing oligonucleotide probes was not increased by paclitaxel. Extract binding activity was judged specific based on competition for binding to a radiolabeled AP-1 probe with increasingly molar excess of unlabeled AP-1 oligonucleotide (Fig. 2B, Lanes B—D), whereas oligonucleotide devoid of an AP-1 site was not an effective competitor (Fig. 2B, Lanes E—G). Nuclear extract binding to the κB and Ets sites was also competed with the corresponding unlabeled oligonucleotides (data not shown).

On the basis of these findings, we sought to characterize the paclitaxel-inducible AP-1 binding activity further. Paclitaxel treatment resulted in a measurable increase in nuclear AP-1 binding activity within 2 h, which was further increased at 4 and 8 h (Fig. 3A). AP-1 binding activity at 16 and 24 h was reduced to a level approximating that of control cells. Paclitaxel also induced AP-1 binding activity in a dose-dependent manner (Fig. 3B, Lanes AP-1), with maximal AP-1 binding activity occurring at paclitaxel concentrations of 6 ng/ml or greater. The levels of NF-κB (data not shown) and Ets DNA binding activities in these extracts were not increased by paclitaxel. For comparison with AP-1, Ets binding as a function of paclitaxel concentration is shown in Fig. 3B (Lanes Ets). It should be noted that we observed the migration of a faster nucleoprotein complex during EMSA; however, this complex was not consistently observed in every experiment, and its origin remains unclear.

To identify the factors that make up paclitaxel-inducible AP-1 activity, highly specific Abs directed against individual AP-1 proteins were evaluated for the ability to deplete nuclear extracts of DNA binding activity. The extract used for this analysis was isolated from lymphoblasts treated with 60 ng/ml paclitaxel (8 h). Incubation of nuclear extracts with 5 μg of anti-JunD, anti-JunB, or anti-c-Jun Abs resulted in a reduction of AP-1 binding activity, whereas 5 μg of nonimmune IgG did not affect DNA binding activity in comparison to control nuclear extracts (Fig. 4A). Abs directed against c-Fos, FosB,
Paclitaxel Causes a Transient Increase in JNK Activity. The transcriptional activity of Jun/AP-1 is regulated by the JNK subfamily of MAP kinases via phosphorylation in its NH₂ terminus (27, 30). Several members of the MAP kinase family, including JNK, have been implicated in surface receptor- and γ-irradiation-induced apoptosis in mammalian cells (24). We investigated, therefore, whether paclitaxel might regulate the activities of individual MAP kinases. Western blot analysis of whole-cell extracts from control and paclitaxel-treated RPMI-1788 B lymphoblasts revealed the presence of immunoreactive p46 JNK1, p38 MAP kinase, and p42 ERK2 (Fig. 6). The individual expression profiles for JNK1, p38 MAP kinase, and ERK2 were similar in control and at several times after paclitaxel treatment, indicating that paclitaxel does not affect the cellular levels of these MAP kinases. Of note, under the conditions used to prepare whole-cell extracts and subsequent Western blot, ERK1 and JNK2 and Fra1 proteins had little, if any, measurable effect on AP-1 binding activity. As a control for the efficacy of these Abs, anti-c-Fos, anti-FosB, and anti-Fra1 Abs were effective at depleting AP-1 binding activity in nuclear extracts isolated from mature B cells (Fig. 4B).

AP-1-dependent Gene Transcription Is Increased in Response to Paclitaxel. To determine whether the paclitaxel-induced AP-1 activity is capable of stimulating de novo gene transcription in vivo, we used a series of chimeric gene plasmids that contained tandem copies of TREs coupled to a minimal herpes simplex virus-tk promoter-CAT reporter fusion gene (39). Induction of CAT gene transcription requires binding of Jun homodimers or Jun/Fos heterodimers to the TRE (39). RPMI-1788 B lymphoblasts were transiently transfected with TRE-CAT plasmids and treated with paclitaxel. As a positive control for these studies, cells were stimulated with 100 ng/ml PMA, which induces AP-1 activity and TRE-dependent gene expression in several cell types, including B cells (39, 42). Paclitaxel stimulated a 2- and 14-fold increase in 3X TRE-CAT and 5X TRE-CAT activity above control cells, respectively (Fig. 5). By comparison, PMA stimulated a similar increase in CAT reporter activity (Fig. 5), whereas transfection of cells with an identical CAT reporter gene plasmid devoid of TREs was not induced by paclitaxel or PMA (Fig. 5, pCAT).

Fig. 3. Paclitaxel induces AP-1 binding activity in a dose- and time-dependent manner. A, cells were treated with 60 ng/ml paclitaxel for the indicated times. B, cells were cultured for 8 h in the presence of varying concentrations of paclitaxel. Nuclei were then isolated and examined for binding to the AP-1 (A and B) or Ets (B) probes. Lane P, migration of the respective probes alone. Arrows, migration of specific nucleoprotein complexes.

Fig. 4. Immunoreactive Jun proteins are present in paclitaxel-induced AP-1. A, nuclear extracts isolated from RPMI-1788 cells at 8 h following paclitaxel treatment were incubated with Abs directed against JunD, c-Jun, JunB, c-Fos, FosB, or Fra1 proteins plus protein G-agarose, as outlined for “Materials and Methods.” The immune complexes were removed, and the depleted nuclear extracts were examined for AP-1 binding. Lane Control, AP-1 binding activity of nuclear extracts incubated in the absence of added Ab; Lane IgG, extracts immunodepleted with nonimmune IgG; Lane Probe, migration of the oligonucleotide. B, mature Balb/c B cells were stimulated via the B-cell antigen receptor complex for 90 min as described (38). Nuclear extracts were isolated and incubated with nonimmune IgG, anti-c-Fos, anti-FosB, or anti-Fra1 Abs plus protein G-agarose, as outlined for A. The depleted nuclear extracts were examined for AP-1 binding activity, as described in A. Arrow, position of AP-1 nucleoprotein complexes.

Fig. 5. Paclitaxel stimulates TRE-dependent gene transcription. Cells were transiently transfected with 10 μg of the indicated plasmids. After 24 h, cells were cultured in medium alone or treated with 30 ng/ml paclitaxel (B) or 100 ng/ml PMA (D). CAT activity was measured as described in “Materials and Methods.” The data are presented as the mean fold induction of CAT activity and are representative of three independent experiments (bars, SE).

Fig. 6. Expression of MAP kinases in RPMI-1788 B lymphoblasts. Cells were cultured in medium (M) alone or treated with 60 ng/ml paclitaxel for 1, 2, 5, and 24 h. Detergent-soluble cell extracts were prepared, subjected to SDS-PAGE, and immunoblotted with anti-p46 JNK (JNK1), anti-ERK2 (ERK2) and anti-p38 MAP kinase (p38 MAP) Abs. Lane D, control cells treated with DMSO alone for 5 h.
STIMULATION OF THE JNK/AP-1 PATHWAY BY PACLITAXEL

A.

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Fig. 7. Paclitaxel transiently elevates JNK activity in RPMI-1788 lymphoblasts. A, RPMI-1788 lymphoblasts were cultured in medium alone (M), medium containing 60 ng/ml paclitaxel for 5, 10, 30, and 60 min and 5 and 24 h, or 100 ng/ml phorbol ester (PMA) for 10 and 30 min. B, Jurkat T cells were cultured in medium alone (M), medium containing 60 ng/ml paclitaxel for 10 and 30 min, or 10 ng/ml phorbol ester (PMA) for 10, 30, and 60 min. JNK activity was measured by solid-phase kinase assays using GST-cJun as substrate, as described in "Materials and Methods." 43 kDa, M, 43,000. Arrows, phosphorylated forms of GST-cJun. Note that exposure of cells to DMSO did not affect JNK activity (data not shown).

were not detected, suggesting that these specific MAP kinases may not be expressed in RPMI-1788 B lymphoblasts.

To determine whether JNK activity was affected by paclitaxel, we used a GST-cJun (1–89) fusion protein bound to GSH-Sepharose beads to precipitate JNK or JNK-like activities from RPMI-1788 B lymphoblast lysates. The precipitated complex was subjected to in vitro solid-phase kinase assay, and then phosphorylation on Ser-63 was measured by immunoblotting with anti-phospho(Ser-63)-c-Jun Ab. Phosphorylation of the GST-cJun fusion protein was detected in whole-cell lysates isolated from control cells (Fig. 7A). Paclitaxel stimulated a rapid and significant increase in Ser-63 phosphorylation at 5 and 10 min. The level of GST-C-Jun phosphorylation was similar to that of control cells when measured at 30 min. It should be noted that JNK activity was decreased at 24 h compared to control cells; however, we believe this reflects a decrease in total cellular JNK1 protein observed (see Fig. 6). As a positive control for these experiments, treatment of lymphoblasts with 100 ng/ml phorbol ester led to an increased phosphorylation of GST-C-Jun at 30 min, with three distinct phosphorylated forms of GST-cJun appearing (Fig. 7A, arrows). Interestingly, we found that paclitaxel treatment of human Jurkat T cells was accompanied by an increased phosphorylation of JNK substrate GST-cJun at 10 and 30 min (Fig. 7B). Exposure of Jurkat T cells to phorbol ester also led to increased GST-cJun phosphorylation, with a second prominent phosphorylated form appearing at 30 and 60 min (Fig. 7B, arrows).

ERK Activity is Transiently Decreased in Response to Paclitaxel.

To determine whether paclitaxel might affect ERK activity, ERK immune complexes were isolated from control and paclitaxel-treated cells and subjected to in vitro solid-phase kinase assay using GST-Elk1 fusion protein as substrate. The phosphorylation of Elk1 at Ser-383 was then measured by immunoblotting with anti-phospho(Ser-383)-Elk1 Ab (43–45). Control RPMI-1788 B lymphoblasts expressed significant ERK-mediated GST-Elk1 phosphotransferase activity (Fig. 8A). Paclitaxel treatment resulted in a marked decrease in GST-Elk1 phosphorylation within 5 min, which was sustained for at least 60 min. An increase in the level of GST-Elk1 phosphorylation was detected at 5, 8, and 16 h and returned to a level equal to that of control cells at 24 h. By comparison, treatment of lymphoblasts with phorbol ester for 30 min did not result in a detectable change in the basal level of GST-Elk1 phosphorylation (Fig. 8A).

p38 MAP kinase is activated by dual phosphorylation on Thr-180 and Tyr-182 in response to a variety of cellular stresses, including inflammatory cytokines and osmotic shock (35, 36). We sought to determine whether paclitaxel affected the phosphorylation status of p38 MAP kinase by immunoblotting cellular extracts with an anti-phospho(Thr-180/Tyr-182)p38 MAP kinase Ab. As shown in Fig. 8B, a low level of phosphorylated p38 MAP kinase was detected in control RPMI-1788 lymphoblasts; however, paclitaxel did not affect the level of p38 MAP kinase phosphorylation at the time points examined. In control experiments conducted in parallel, it was observed that osmotic shock and endotoxic LPS caused a marked increase in the phosphorylation at Thr-180/Tyr-182 in the RPMI-1788 lymphoblast. As an additional control for these studies, p38 MAP kinase phosphorylated at Thr-180/Tyr-182 was increased in cellular extracts from C-6 glioma cells following exposure to the stress-inducing agent anisomycin.

DISCUSSION

In this study, we found that paclitaxel treatment of RPMI-1788 B lymphoblast cells led to an increase in nuclear AP-1 binding activity in a time- and dose-dependent manner. The induction of AP-1 binding activity was selective, insofar as basal DNA binding activities for the inducible nuclear factors Ets and NF-κB were not affected by paclitaxel at the doses used and the time points examined in this study. Although our analysis of trans-acting DNA-binding proteins is limited, these findings suggest that paclitaxel is not "globally" perturbing inducible nuclear factor activity (48). Evidence for the presence of Jun proteins in paclitaxel-induced AP-1 binding activity was demonstrated by highly specific anti-JunD, anti-JunB, and anti-c-Jun Abs to deplete nuclear extracts of DNA binding activity. By contrast, Abs directed against c-Fos, FosB, and Fra1 proteins did not deplete AP-1 activity. Although the presence of Fos family or Fos-like proteins in paclitaxel-induced AP-1 binding activity cannot be ruled out completely, we found that these Abs were effective at depleting AP-1 binding activity in nuclear extracts from activated mature B cells (42).

We also demonstrated that the paclitaxel-induced AP-1 binding activity is biologically functional in vivo. This conclusion is based on the observation that paclitaxel stimulated transcription of a CAT

Fig. 8. Paclitaxel transiently decreases ERK activity in RPMI-1788 lymphoblasts. A, RPMI-1788 lymphoblasts were cultured in medium alone (M) or treated with 60 ng/ml paclitaxel for 5, 10, 30, and 60 min and 5, 8, 16, and 24 h. ERK activity was measured using a GST-Elk1 as substrate as described in "Materials and Methods." Lane PMA, level of phospho ERK1/2 proteins in control RPMI-1788 lymphoblasts; however, paclitaxel did not affect the level of p38 MAP kinase phosphorylation at the time points examined. In control experiments conducted in parallel, it was observed that osmotic shock and endotoxic LPS caused a marked increase in the phosphorylation at Thr-180/Tyr-182 in the RPMI-1788 lymphoblast. As an additional control for these studies, p38 MAP kinase phosphorylated at Thr-180/Tyr-182 was increased in cellular extracts from C-6 glioma cells following exposure to the stress-inducing agent anisomycin.

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reporter gene, the activity of which is TRE dependent. In fact, the stimulation of reporter gene transcription by paclitaxel was comparable to the fold induction observed in RPMI-1788 B lymphoblasts stimulated with phorbol diester, a strong inducer of AP-1 activity (39). The ability of Jun/AP-1 to activate transcription is modulated through phosphorylation of the NH2-terminal transactivating domain by the JNK/AP-1 in paclitaxel-induced cell death. A number of studies suggest that AP-1 and JNK are essential in some forms of apoptosis, namely cell death, which requires de novo protein synthesis (52); however, they are not apparently involved in Fas- and tumor necrosis factor-induced apoptosis (53, 54). In experiments not presented herein, we have found that the paclitaxel-induced AP-1 binding activity is blocked by cycloheximide. In light of the aforementioned studies and the finding that paclitaxel promotes the activation of JNK, it is intriguing to consider that AP-1 may be needed for de novo transcription of genes, the products of which are required further “downstream” in the cell death pathway.

In contrast to the JNK/AP-1 response, p38 MAP kinase activation as monitored by phosphorylation at Thr-180 and Tyr-182 was not affected by paclitaxel; however, phosphorylation of p38 MAP kinase was rapidly increased in RPMI-1788 lymphoblasts by osmotic shock and endotoxin LPS. Thus, although p38 MAP kinase can be activated via phosphorylation in RPMI-1788 lymphoblasts by conditions known to induce cellular stress responses and p38 MAP kinase activity in many mammalian cell types (24, 35, 55), paclitaxel does not apparently affect its activation.

ERK2 activity was detected in control RPMI-1788 lymphoblasts. Paclitaxel caused a rapid decrease in ERK2 activity, which was sustained for at least 60 min. Although the biological significance of the reduced ERK2 activity is presently unknown, it is important to note that ERK2 activity was not decreased in RPMI-1788 lymphoblasts stimulated with phorbol ester for 30 min. Moreover, phosphorylation of the ERK2 substrate GST-Elk1 increased between 5 and 16 h, returning to the level observed in control cells by 24 h. The recovery of ERK2 activity may be needed for a distinct signal transduction pathway(s) in vivo that targets different “downstream” substrates in response to paclitaxel. For example, Neckers and coworkers (10, 12) found that paclitaxel stimulated a c-ras-1/ERK pathway in human MCF7 breast cancer cells when measured at 16 h. ERKs have been shown to be bound by the microtubule cytoskeleton in activated cells (56), and evidence is accumulating for a role of c-ras-1/ERK components in the inactivation of bcl-2 by phosphorylation (12, 15, 57).

In summary, we show that paclitaxel causes the transient activation of the JNK/AP-1 pathway and down-regulation of ERK2 activity in RPMI-1788 B lymphoblasts. These findings suggest that multiple MAP kinase signal transduction pathways may be involved in the early cellular response of RPMI-1788 B lymphoblasts to paclitaxel. Additional studies should provide insight into the biological significance of JNK/AP-1 and ERK2.

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