

Induction of Apoptosis in Human Leukemia Cells by Grape Seed Extract Occurs via Activation of c-Jun NH₂-Terminal Kinase

Ning Gao,^{1,2} Amit Budhraj,² Senping Cheng,² Hua Yao,² Zhuo Zhang,² and Xianglin Shi²

Abstract Purpose: To characterize the functional role of c-Jun NH₂-terminal kinase (JNK) and other apoptotic pathways in grape seed extract (GSE)-induced apoptosis in human leukemia cells by using pharmacologic and genetic approaches.

Experimental Design: Jurkat cells were treated with various concentrations of GSE for 12 and 24 h or with 50 μg/mL GSE for various time intervals, after which apoptosis, caspase activation, and cell signaling pathways were evaluated. Parallel studies were done in U937 and HL-60 human leukemia cells.

Results: Exposure of Jurkat cells to GSE resulted in dose- and time-dependent increase in apoptosis and caspase activation, events associated with the pronounced increase in Cip1/p21 protein level. Furthermore, treatment of Jurkat cells with GSE resulted in marked increase in levels of phospho-JNK. Conversely, interruption of the JNK pathway by pharmacologic inhibitor (e.g., SP600125) or genetic (e.g., small interfering RNA) approaches displayed significant protection against GSE-mediated lethality in Jurkat cells.

Conclusions: The result of the present study showed that GSE induces apoptosis in Jurkat cells through a process that involves sustained JNK activation and Cip1/p21 up-regulation, culminating in caspase activation.

The hematologic malignancies constitute a group of cancers that arise from malignant transformation of various cells derived from peripheral blood, lymphatic system, and bone marrow. These diseases include the acute and chronic leukemias, Hodgkin's disease (now termed Hodgkin's lymphoma), non-Hodgkin's lymphoma, and multiple myeloma. The heterogeneity seen in this collection of cancers reflects the complexity of the normal hematopoietic and immune systems.

Individually, these cancers are less common than some solid tumors; however, collectively, leukemia, lymphoma, and myeloma accounted for an estimated 118,310 new cancer cases in 2006 (~9% of cancer cases diagnosed in the United States) and 53,920 cancer deaths, placing this group fourth among cancers in each category (1). Established causes of leukemia include occupational exposure to ionizing radiation (2), certain drugs used in the treatment of cancer (3), and some chemicals (most notably benzene) used largely in industrial settings (4). Because of an increase in the morbidity and mortality of human

leukemia in recent years, control of human leukemia through chemoprevention or intervention is highly desirable.

Epidemiologic studies have indicated that consumption of a fruit and vegetable-based diet reduces the risk of various cancers (5). Due to these observations, the latest global strategy on the prevention of cancer recommends consumption of colorful fruits and vegetables (6). Consequently, the focus of cancer research in recent years has been shifting toward the isolation and characterization of potential chemopreventive agents present in fruits and vegetables (7). Grape seed extract (GSE) contains mainly phenols such as proanthocyanidins, which have shown promising chemopreventive and/or anticancer efficacy in various cell culture and animal models (8). It has been shown that GSE reduces the incidence of carcinogen-induced mammary tumors in rats and skin tumors in mice and inhibits the growth of human cancer cells of various phenotypes *in vitro* and *in vivo* (9–14). GSE exhibits cytotoxicity toward some cancer cells such as skin (15), breast (16), colon (17), lung (9), gastric (9), and prostate (11) cancers while enhancing the growth and viability of normal cells (18). GSE lethality is regulated by multiple mechanisms including inactivation of cytoprotective pathways such as phosphatidylinositol 3-kinase/protein kinase B (17), activation of stress-related pathway (12), activation of Chk2 and p53 (19), mitochondrial damage leading to cytochrome *c* and apoptosis-inducing factor release (11), inhibition of nuclear factor-κB activity (20), among others. GSE also inhibits cell growth, induces G₁-phase cell cycle arrest and apoptosis, and modulates cell cycle regulators with a strong effect for Cip1/p21 up-regulation in human colorectal cancer cells (21).

Recently, GSE has been reported to induce apoptosis in human leukemia cells by activation of caspases such as

Authors' Affiliations: ¹Department of Pharmacognosy, School of Pharmacy, 3rd Military Medical University, Chongqing, People's Republic of China and ²Graduate Center for Toxicology, College of Medicine, University of Kentucky, Lexington, Kentucky

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Requests for reprints: Xianglin Shi, Graduate Center for Toxicology, College of Medicine, University of Kentucky, Lexington, KY 40536-0305. Phone: 859-257-4054; Fax: 859-323-1059; E-mail: xshi5@email.uky.edu.

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Translational Relevance

The hematologic malignancies constitute a group of cancers that arise from malignant transformation of various cells derived by peripheral blood, lymphatic system, and bone marrow. Because of increase in the morbidity and mortality of human leukemia in recent years, control of human leukemia through chemoprevention or intervention is highly desirable. The present study has provided evidence that GSE induces human leukemia cell death with the activation of caspase-3, -8, and -9 as well as PARP cleavage and that GSE-induced apoptosis is preceded by the activation of JNK and up-regulation of Cip1/p21. The data presented here suggest that Cip1/p21 and JNK signaling pathway may represent attractive targets for GSE-induced apoptosis in human leukemia cells. The results of this study could have implications for the incorporation of agents such as GSE into the chemopreventive or therapeutic intervention against leukemia and possibly other hematologic malignancies.

caspase-3 (22). However, the detailed molecular mechanism of GSE-induced apoptosis in human leukemia cells has not yet been explored. The purpose of the present study was to characterize the functional role of c-Jun NH₂-terminal kinase (JNK) and related cell signaling pathways, using pharmacologic and genetic approaches, on the lethality of GSE toward human leukemia cells. Our results indicate that activation of JNK plays a key role in mediating the cytotoxic effects of GSE in these cells and suggest that interruption of the JNK pathway can dramatically potentiate GSE-related antileukemic actions. It also indicates a hierarchical model of GSE-induced lethality in human leukemia cells characterized by activation of JNK pathway, leading to up-regulation of Cip1/p21 and culminating in caspase activation and apoptosis.

Materials and Methods

Chemicals and reagents. GSE-standardized preparation was obtained as a gift from its commercial vender, Kikkoman. The details of GSE preparation from grape seeds were described recently (23) and the chemical constituents present within this GSE preparation were analyzed by Yamakoshi et al. (23) and include 89.3% (w/w) procyanidins, 6.6% monomeric flavonols, 2.24% moisture content, 1.06% protein, and 0.8% ash. GSE was dissolved in sterile DMSO at a stock concentration of 50 mg/mL and stored at -20°C. In all of the experiments, the final concentration of DMSO did not exceed 0.1% (v/v). Same volume of DMSO was used as a negative control. SP600125 and Z-VAD-FMK were purchased from EMD Biosciences. Antibodies against Bad, Akt, phospho-extracellular signal-regulated kinase (ERK), ERK, phospho-JNK, JNK, and β -actin were purchased from Santa Cruz Biotechnology. Cleaved caspase-3, cleaved caspase-9, Bcl-xL, phospho-Akt, phospho-mTOR, mTOR, phospho-p38, and p38 were purchased from Cell Signaling. XIAP, Mcl-1, Bax, and Cip1/p21 were purchased from Pharmingen. Poly(ADP-ribose) polymerase (PARP) was purchased from Biomol. Caspase-8 was purchased from Alexis. Bcl-2 was purchased from DAKO.

Cell culture and transfection. U937, HL-60, and Jurkat human leukemia cells were obtained from the American Type Culture

Collection and cultured in RPMI 1640 supplemented sodium pyruvate, sodium bicarbonate, nonessential amino acid, L-glutamine, penicillin, streptomycin, and 10% fetal bovine serum. Jurkat cells (1.5×10^6) were transiently transfected with 1 μ g JNK1- annealed dsRNAi oligonucleotide 5'-CGUGGGAUUUAUGGUCUGUGT-3'/3'-TTGCACCUAAAUAC-CAGACAC-5' (Orbigen) using the Amaxa Nucleofector as recommended by the manufacturer. After incubation at 37°C and 5% CO₂ for 24 h, transfected cells were treated with GSE and subjected to determination of apoptosis and Western blot. The epitope-tagged JNK1 (kindly provided by Dr. Roger J. Davis, University of Massachusetts Medical School) was cloned into the mammalian expression vector pcDNA3 (Invitrogen). Jurkat cells were stably transfected with a JNK1 expression vector, and stable single-cell clones were selected in the presence of 400 μ g/mL geneticin.

Annexin V/propidium iodide assays for apoptosis. For Annexin V/propidium iodide (PI) assays, cells were stained with Annexin V-FITC and PI and then evaluated for apoptosis by flow cytometry according to the manufacturer's protocol (BD Pharmingen). Briefly, 1×10^6 cells were washed twice with cold PBS and stained with 5 μ L Annexin V-FITC and 10 μ L PI (5 μ g/mL) in $1 \times$ binding buffer [10 mmol/L HEPES (pH 7.4), 140 mmol/L NaOH, 2.5 mmol/L CaCl₂] for 15 min at room temperature in the dark. The apoptotic cells were determined using a Becton Dickinson FACScan cytofluorometer. Both early apoptotic (Annexin V-positive, PI-negative) and late apoptotic (Annexin V-positive and PI-positive) cells were included in cell death determinations.

Western blot analysis. Western blot analysis was done using the NuPAGE Bis-Tris electrophoresis system (Invitrogen). The total cellular samples were washed twice with cold PBS and lysed in $1 \times$ NuPAGE LDS sample buffer supplemented with 50 mmol/L DTT (Fisher Biotech). The protein concentration was determined using Coomassie Protein Assay Reagent (Pierce). The total cellular protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membrane in 20 mmol/L Tris-HCl (pH 8.0) containing 150 mmol/L glycine and 20% (v/v) methanol. Membranes were blocked with 5% fat-free dry milk in $1 \times$ TBS containing 0.05% Tween 20 and incubated with antibodies. Protein bands were detected by incubation with horseradish peroxidase-conjugated antibodies (Kirkegaard and Perry Laboratories) and visualized with enhanced chemiluminescence reagent (Perkin-Elmer Life Sciences).

Statistical analysis. For analysis of apoptosis, values were presented as mean \pm SD. Statistical differences between control and treated groups were determined by Student's *t* test. Differences were considered statistically significant for $P < 0.05$ or $P < 0.01$.

Results

GSE induced apoptosis and caspase activation in dose- and time-dependent manners in Jurkat cells. A dose-response analysis of GSE-mediated Jurkat cells revealed a moderate increase in apoptosis 12 and 24 h after exposure to GSE at concentration of 10 μ g/mL ($P < 0.01$ versus control) and very extensive apoptosis at concentrations ≥ 25 μ g/mL ($P < 0.01$ versus control; Fig. 1A). A time-course study of cells exposed to 50 μ g/mL GSE showed a significant increase in apoptosis as early as 4 h after drug exposure ($P < 0.01$ versus control). These events became apparent after 12 h of drug exposure and reached near-maximal levels after 24 h ($P < 0.01$ versus control; Fig. 1B).

Western blot analysis revealed that exposure of Jurkat cells to 10 μ g/mL GSE resulted in a slight increase in cleavage/activation of caspase-3, -8, and -9 as well as PARP degradation and a marked increase at concentrations ≥ 25 μ g/mL (Fig. 1C). A time-course study of cells exposed to 50 μ g/mL GSE revealed marked increases in cleavage/activation of caspase-3, -8, and -9

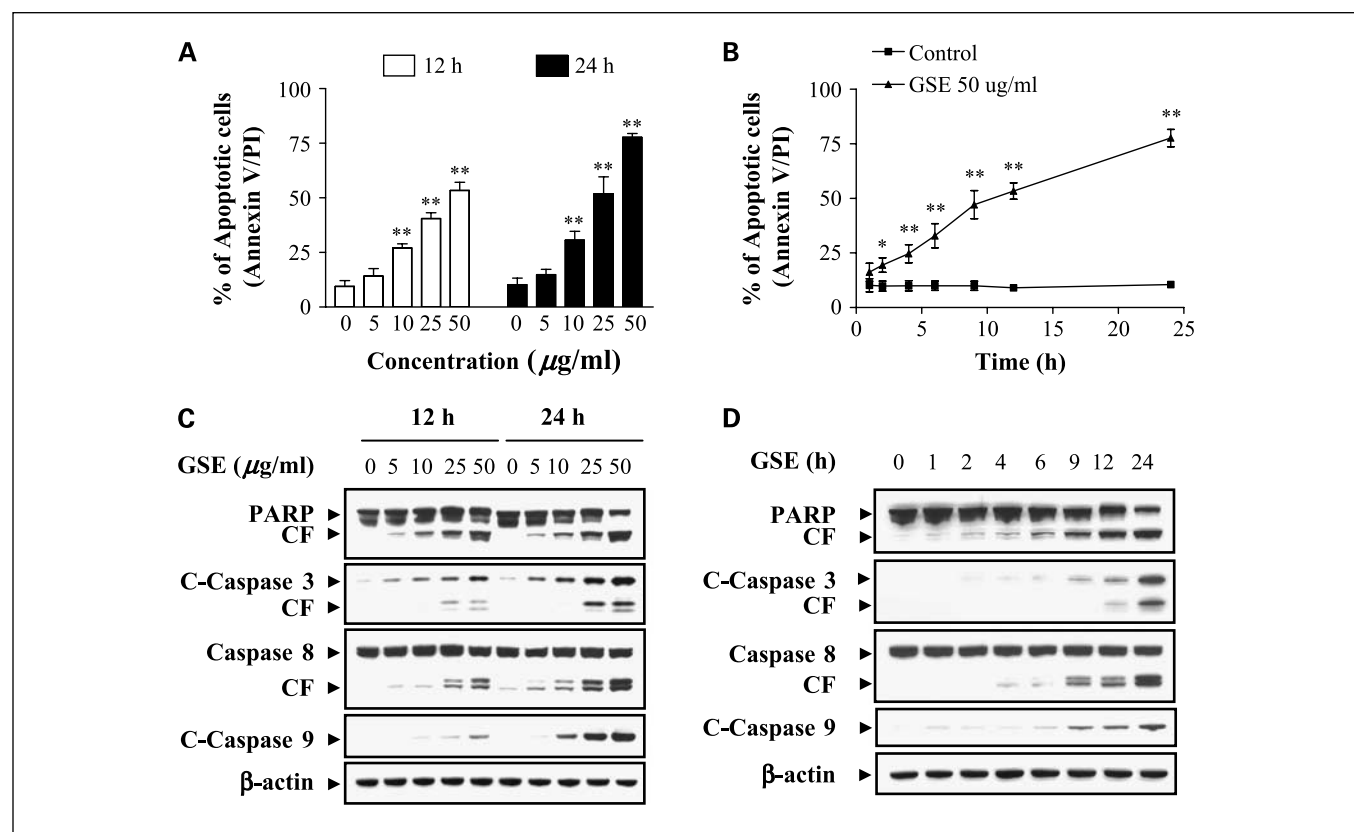


Fig. 1. GSE markedly induces apoptosis in Jurkat human leukemia cells in dose- and time-dependent manners. *A*, Jurkat cells were treated without or with various concentrations of GSE as indicated for 12 and 24 h. *B*, cells were treated with 50 µg/mL GSE for various time intervals as indicated. For *A* and *B*, cells were stained with Annexin V/PI, and apoptosis was determined using flow cytometry as described in Materials and Methods. Mean \pm SD of three separate experiments (obtained from Annexin V assays). *, $P < 0.05$; **, $P < 0.01$, values for cells treated with GSE were significantly increased compared with values in control cells (Student's *t* test). *C*, cells were treated without or with various concentrations of GSE as indicated for 12 and 24 h. *D*, cells were treated without or with 50 µg/mL GSE for 1, 2, 4, 6, 9, 12, and 24 h. After treatment in *C* and *D* with the indicated GSE concentrations or the indicated intervals, total cellular extracts were prepared and subjected to Western blot assay using antibodies against PARP, cleaved caspase-3, caspase-8, and cleaved caspase-9. For Western blot analysis, each lane was loaded with 30 µg protein. Blots were subsequently stripped and re-probed with antibody against β -actin to ensure equivalent loading and transfer. Two additional studies yielded equivalent results.

as well as PARP degradation 12 and 24 h after drug exposure (Fig. 1D).

Exposure of human leukemia cells to GSE resulted in increased expression of Cip1/p21 but had no effects on levels of Bcl-2 family proteins. Dose- and time-dependent effects of GSE were then evaluated in relation to expression of various Bcl-2 family members and cell cycle regulatory proteins. A dose-dependent study showed that exposure of Jurkat cells to varying concentration of GSE did not discernibly modify the expression of Bcl-2, Bcl-xL, XIAP, Mcl-1, Bax, and Bad (Fig. 2A). A time-course study also showed that exposure of Jurkat cells to 50 µg/mL GSE for various intervals did not appreciably modify the expression of these proteins (Fig. 2B). In contrast, Western blot analysis revealed a strong dose-dependent increase in expression of Cip1/p21 12 and 24 h after exposure to GSE (Fig. 2C). A time-course study showed that exposure of Jurkat cells to 50 µg/mL GSE resulted in marked increase in expression of Cip1/p21 as early as 4 h after drug exposure (Fig. 2D).

Exposure of human leukemia cells to GSE resulted in a pronounced increase in levels of phospho-JNK but did not affect levels of phospho-Akt, phospho-ERK, or phospho-p38. Effects of treatment with GSE on expression of survival and stress-

related signaling pathways were examined next. Western blot analysis indicated that exposure of Jurkat cells to GSE resulted in a dose-dependent increase in levels of phospho-JNK but had no significant effects on total JNK (Fig. 3A). A time-course study showed that exposure of Jurkat cells to 50 µg/mL GSE resulted in marked increase in levels of phospho-JNK as early as 4 h after drug exposure and reached near-maximal levels at 24 h (Fig. 3B). In contrast, GSE had little or no effect on expression of total or phospho-Akt, ERK, or p38 mitogen-activated protein kinase. These results suggest that reciprocal activation of the stress-related JNK pathway may play an important role in GSE-induced apoptosis.

GSE had similar effects on apoptosis, caspase activation, PARP degradation, Cip1/p21 up-regulation, and JNK activation in U937 and HL-60 human leukemia cells. To determine whether these events were restricted to myeloid leukemia cells, parallel studies were done in U937 (acute myeloid leukemia cells) and HL-60 (acute promyelocytic leukemia cells). These cells exhibited apoptotic effects of GSE similar to those observed in Jurkat cells, although U937 and HL-60 cells are less sensitive than Jurkat cells in GSE-induced apoptosis ($P < 0.01$, compared with control; Fig. 4A). Also, U937 and HL-60 cells exhibited comparable degrees of caspase-3, -8, and -9 activation and

PARP degradation (Fig. 4B). As in the case of Jurkat cells, GSE induced Cip1/p21 expression in U937 and HL-60 cells (Fig. 4C) but had little or no effect on expression of Bcl-1, Bcl-xL, XIAP, Mcl-1, Bax, and Bad in U937 and HL-60 cells (data not shown). Lastly, the ability of GSE to trigger activation of JNK in U937 and HL-60 cells was identical to effects observed in Jurkat cells (Fig. 4D). The results indicate that the effects of GSE are not cell type specific.

GSE lethality was associated with the caspase-independent activation of JNK and Cip1/p21 expression. To assess whether GSE-induced activation of JNK and Cip1/p21 expression are dependent on caspase activation, the pan-caspase inhibitor Z-VAD-FMK was used. Addition of Z-VAD-FMK blocked GSE-induced apoptosis (values for cells treated with both Z-VAD-FMK and GSE were significantly reduced compared with values obtained by GSE alone by Student's *t* test; $P < 0.01$; Fig. 5A), caspase-3, -8, and -9 activation, and PARP degradation (Fig. 5B) but had no effect on Cip1/p21 expression mediated by GSE (Fig. 5C). Z-VAD-FMK also failed to prevent JNK activation induced by GSE (Fig. 5D). Together, these findings indicate that GSE-induced JNK activation and Cip1/p21 up-regulation represent primary rather than caspase-dependent events, suggesting that these events may be involved in GSE-mediated caspase activation and lethality.

JNK activation plays an important functional role in GSE-induced Cip1/p21 up-regulation, caspase activation, and apoptosis. The functional significance of JNK activation in GSE lethality

was then investigated using both pharmacologic and genetic approaches. Coadministration of the JNK inhibitor SP600125 essentially abrogated GSE-mediated apoptosis ($P < 0.01$, compared with GSE treatment alone), caspase-3, -8, and -9 activation, and PARP degradation (Fig. 6A and B). Coadministration of SP600125 also blocked GSE-induced Cip1/p21 expression and JNK activation (Fig. 6C and D). Because SP600125 is not completely specific for JNK (24), a genetic approach using JNK1 small interfering RNA (siRNA) was employed. As shown in Fig. 6E, transient transfection of Jurkat cells with JNK1 siRNA reduced expression of JNK1 to one-fourth compared with control cells and resulted in a significant reduction in GSE-mediated apoptosis ($P < 0.01$ versus control siRNA). To further assess the functional significance of JNK activation in GSE-mediated apoptosis and caspase activation, Jurkat cells ectopically expressing epitope-tagged JNK1 were employed. As shown in Fig. 6F, enforced activation of JNK markedly enhanced GSE-induced apoptosis (10 $\mu\text{g}/\text{mL}$; 24 h) compared with that in vector control cells ($P < 0.01$). Consistent with these findings, GSE was considerably more effective in triggering PARP degradation and caspase cleavage/activation in JNK1-overexpressing cells compared with vector control cells. Western blot analysis documented marked increase in level of total JNK in JNK1-expressing cells, and GSE markedly induced the phosphorylation of JNK in JNK1-expressing cells compared with vector control cells (Fig. 6F). Collectively, these findings indicate that GSE-induced JNK activation plays an important functional

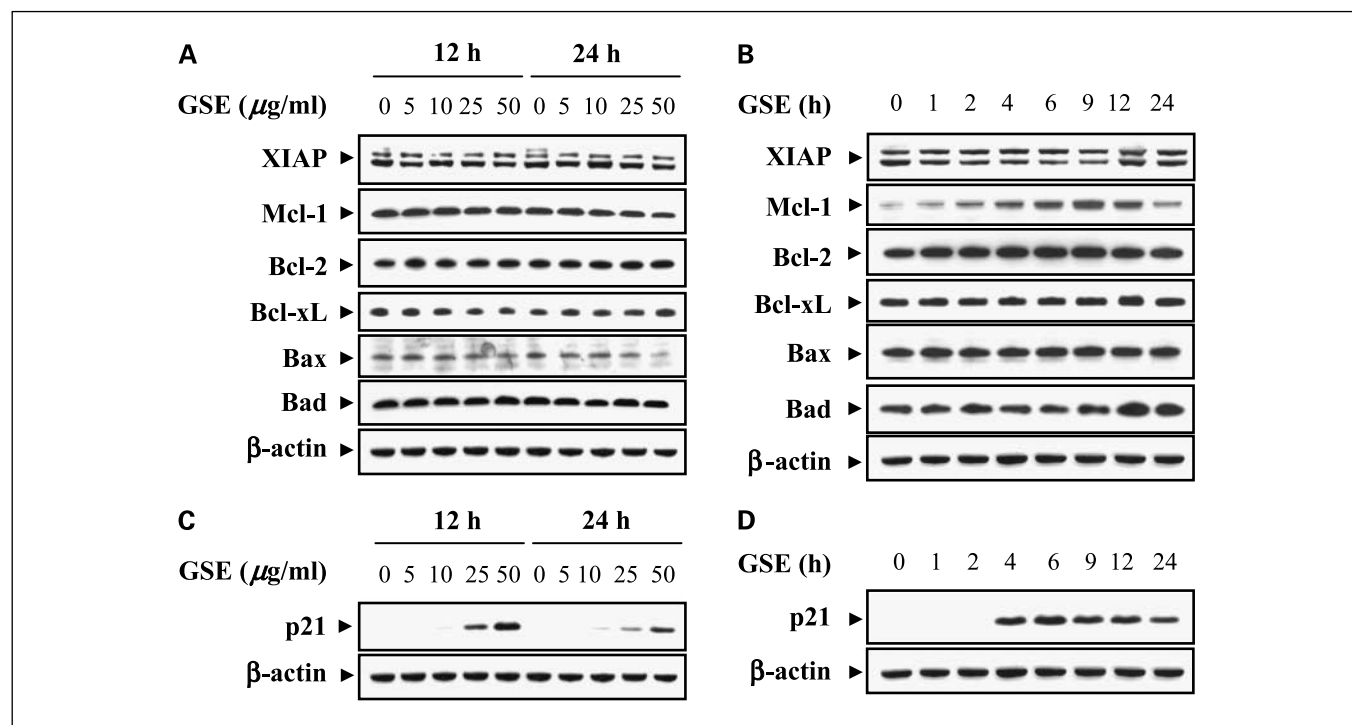


Fig. 2. GSE induces the expression of Cip1/p21 but does not affect the expression of Bcl-2 family members. *A*, Jurkat cells were treated without or with various concentrations of GSE as indicated for 12 and 24 h. *B*, cells were treated without or with 50 $\mu\text{g}/\text{mL}$ GSE for 1, 2, 4, 6, 9, 12, and 24 h. For *A* and *B*, total cellular extracts were prepared and subjected to Western blot analysis using antibodies against Bcl-2 family members including XIAP, Mcl-1, Bcl-2, Bcl-xL, Bax, and Bad. *C*, cells were treated without or with the indicated concentrations of GSE for 12 and 24 h. *D*, cells were treated without or with 50 $\mu\text{g}/\text{mL}$ GSE for 1, 2, 4, 6, 9, 12, and 24 h. For *C* and *D*, total cellular extracts were prepared and subjected to Western blot analysis using antibodies against Cip1/p21 (p21). For Western blot analysis, each lane was loaded with 30 μg protein. Blots were subsequently stripped and reprobed with antibody against β -actin to ensure equivalent loading and transfer. Two additional studies yielded equivalent results.

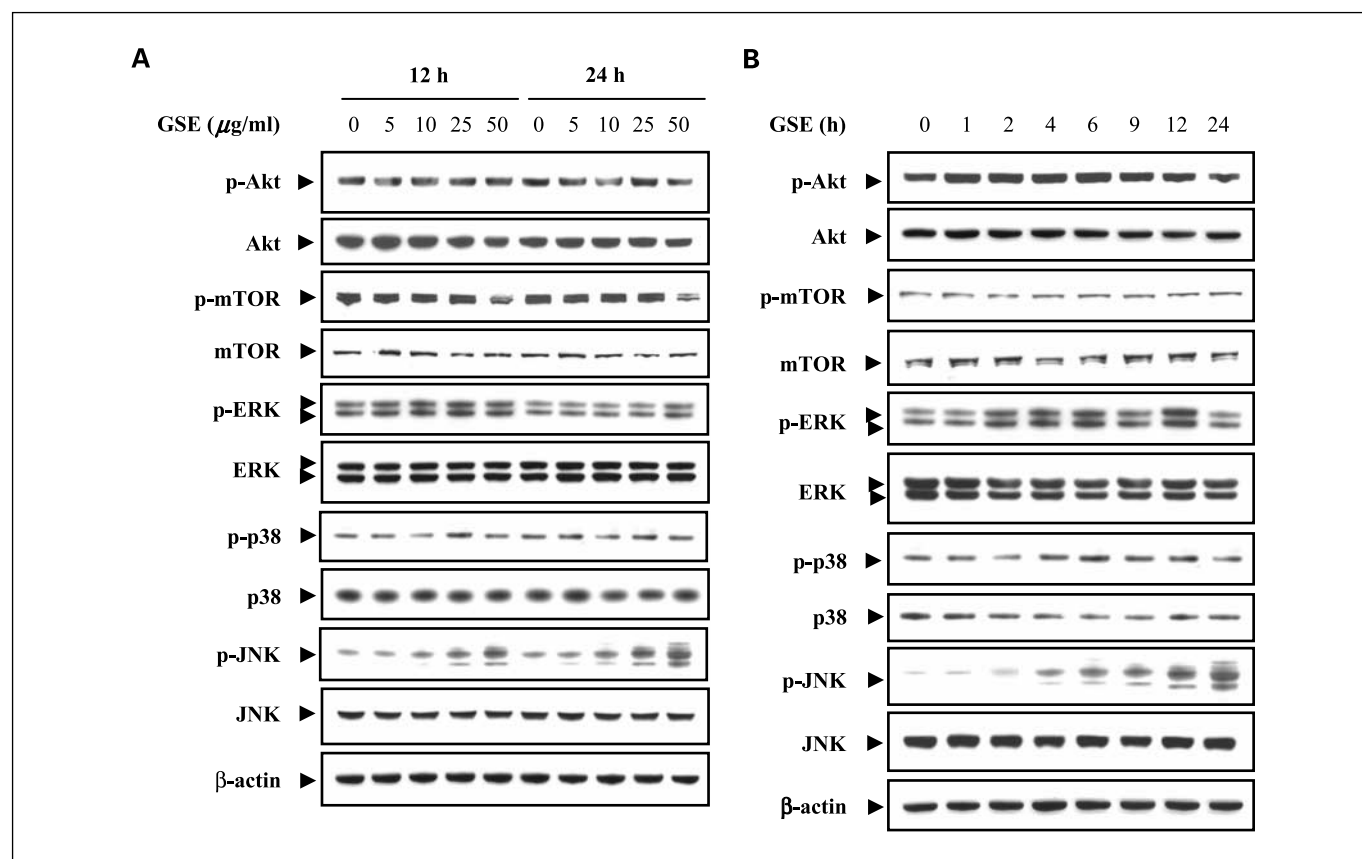


Fig. 3. Effects of GSE on various signaling transduction pathways. *A*, Jurkat cells were treated without or with the indicated concentrations of GSE for 12 and 24 h. *B*, cells were treated without or with 50 $\mu\text{g/ml}$ GSE for 1, 2, 4, 6, 9, 12, and 24 h. Total cellular extracts were prepared and subjected to Western blot analysis using antibodies against phospho-Akt (*p-Akt*), Akt, phospho-ERK (*p-ERK*), ERK, phospho-p38 (*p-p38*), p38, phospho-JNK (*p-JNK*), JNK, phospho-mTOR (*p-mTOR*), and mTOR. For Western blot analysis, each lane was loaded with 30 μg protein. Blots were subsequently stripped and reprobbed with antibody against β -actin to ensure equivalent loading and transfer. Two additional studies yielded equivalent results.

role in GSE-mediated lethality. They also indicate that activation of JNK operates upstream of Cip1/p21 and caspase cleavage/activation in GSE-mediated engagement of the apoptotic cascade.

Discussion

Apoptosis (programmed cell death) is an active process of cell death that takes place under a variety of conditions and is important to induce tumor destruction. It is characterized by distinct morphologic changes and is regulated by a series of biochemical events that lead to cell death. Caspases, a family of aspartate-specific cysteine proteases, which exist as single-chain inactive zymogens, play an important role in the execution phase of apoptosis. "Initiator" caspases, which are long prodomains such as caspase-8 and -9, either directly or indirectly activate "effector" caspases, such as caspase-3 and -7 (25). These effector caspases then cleave intracellular substrates, including PARP, resulting in the dramatic morphologic changes of apoptosis (25). To determine the role of caspases in GSE-induced apoptosis, we examined the activation of caspases by GSE. Treatment of cells with GSE resulted in cleavage/activation of the initiator caspase-8 and -9 and the effector caspase-3 with concomitant induction of apoptosis. Blocking of caspase activation by Z-VAD-FMK, a broad-

spectrum caspase inhibitor, significantly suppressed GSE-induced apoptosis. The activation of caspase-8 in leukemia cells requires association with apoptotic ligands such as tumor necrosis factor- α , Fas ligand, or tumor necrosis factor-related apoptosis-inducing ligand (26). Caspase-9 can be activated by caspase-8 or activated independently by apoptotic protease-activating factor 1 on binding of cytochrome *c* release from the mitochondria (27). The activation of the effector caspase-3 by GSE could then be explained by proteolytic cleavage by these activated upstream caspases. Thus, apoptotic ligand- or mitochondria-mediated activation of the caspase cascade may be a potential mechanism underlying GSE-induced apoptosis in leukemia cells.

The present results also indicate that induction of cell death by GSE in human leukemia cells results in activation of JNK and that this process plays a critical role in regulating the cell death response. Presently, little information is available concerning the functional role of the JNK pathway in mediating GSE-induced lethality, particularly in malignant hematopoietic cells. The results of the present study show that JNK activation plays a key functional role in GSE-mediated caspase activation and subsequent lethality. JNK, also known as stress-activated protein kinases, form an important subgroup of the mitogen-activated protein kinase superfamily. JNK has three isoforms (JNK1, JNK2, and JNK3)

encoded by three different genes. JNK1 and JNK2 are ubiquitous, whereas JNK3 is relatively restricted to brain (28). *In vitro* and gene disruption studies show functional differences among JNK isoforms. JNK1 is the major c-Jun kinase after stimulation, and JNK2 is preferentially bound to c-Jun in unstimulated cells and contributes to c-Jun degradation by an ubiquitin-dependent mechanism. JNK2 also regulates the stability of JunB, c-Myc, and ATF2 (29, 30). The specific molecular targets of JNK include transcription factors AP-1, p53, and c-Myc (31, 32) as well as many other nontranscription factors such as Bcl-2 family members, which are closely related to apoptotic cell death (33). It is known that the involvement of JNK in controlling diverse cellular functions such as cell proliferation (34), differentiation (35), and apoptosis (36) is based on phosphorylation and functional modification of these molecular targets in stimuli-dependent and cell type-dependent manners. In fact, the net balance between cytoprotective (e.g., ERK) and stress-

related (e.g., JNK) signaling may play a critical role in cell survival and death decisions (37). Engagement of the JNK pathway has been shown to play a key functional role in the lethal effects of diverse cytotoxic stimuli, including vinblastine, doxorubicin, and etoposide (38, 39). It is reported that activation of JNK kinase cascade regulated cytochrome *c* release and caspase activation in pramanicin-treated Jurkat cells (40). The finding that pharmacologic and genetic interruption of the JNK pathway attenuated GSE-mediated lethality indicates that stress pathways play a critical functional role in GSE-induced apoptosis. The inhibition of JNK by its specific inhibitor, SP600125, abolished the activation of caspase-3, -8, and -9, PARP cleavage, and apoptosis induced by GSE. The genetic interruption by JNK siRNA also effectively inhibited GSE-mediated activation of caspase-3, -8, and -9, PARP cleavage, and apoptosis.

JNK activity appears to be essentially involved in apoptotic progression of various cell types induced by several different

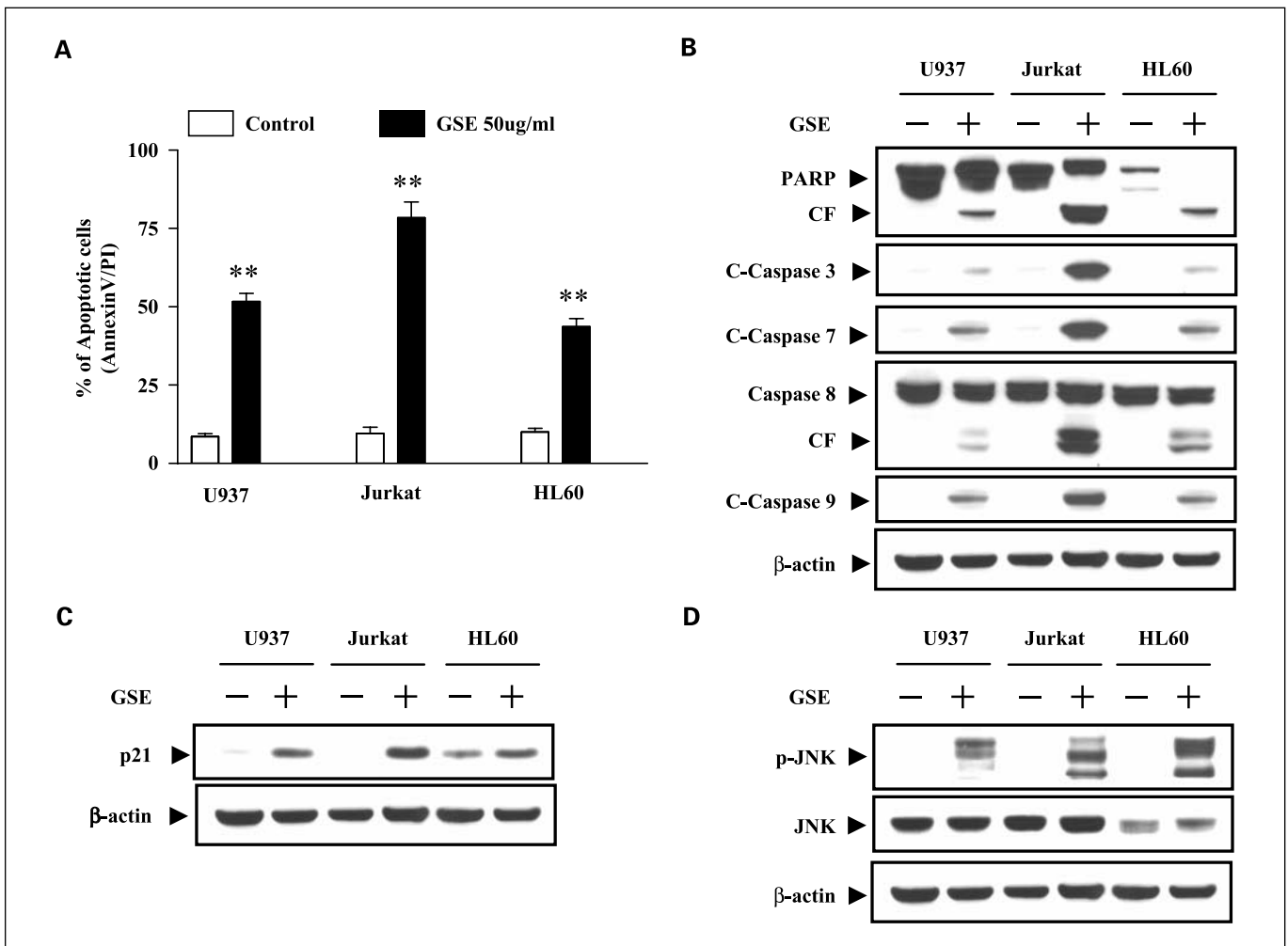


Fig. 4. GSE induces apoptosis in U937, Jurkat, and HL-60 cells. U937, Jurkat, and HL-60 cells were treated without or with 50 μ g/mL GSE for 24 h. **A**, cells were stained with Annexin V/PI, and apoptosis was determined using flow cytometry as described in Materials and Methods. Mean \pm SD of three separate experiments (obtained from Annexin V/PI assays). **, $P < 0.01$, values for cells treated with GSE were significantly increased compared with values in control cells (Student's *t* test). **B**, total cellular extracts were prepared and subjected to Western blot analysis using antibodies against PARP, cleaved caspase-3, caspase-8, and cleaved caspase-9. **C**, total cellular extracts were also prepared and subjected to Western blot assay using antibodies against Cip1/p21. **D**, total cellular extracts were also prepared and subjected to Western blot assay using antibodies against phospho-JNK and JNK. For Western blot analysis, each lane was loaded with 30 μ g protein. Blots were subsequently stripped and reprobbed with antibody against β -actin to ensure equivalent loading. Two additional studies yielded equivalent results.

apoptotic stimuli. JNK activity is regulated by various different mechanisms in cells under the different experimental conditions. A recent study has shown one of the mechanisms by which JNK activation is dependent on activation of the caspase cascade. It is noted that tumor necrosis factor- α and anti-Fas antibody-induced prolonged JNK and ERK and ROS accumulation were completely inhibited by a caspase inhibitor, suggesting that these events can be downstream of the caspase cascade (41, 42). Meanwhile, activation of JNK also operates upstream of mitochondrial injury and caspase activation in stimuli-mediated engagement of the apoptotic cascade. It has been reported that inhibition of JNK activation by either a specific inhibitor of JNK, SP600125, or JNK siRNA abrogated 2-methoxyestradiol-mediated caspase activation and apoptosis (43). Blocking JNK by either dominant-negative mutant (DN-JNK) or cotreatment with a specific JNK inhibitor, SP600125, abrogates both stress-induced release of Smac, activation of

caspases, and induction of apoptosis (44). Therefore, JNK activation in stress-induced cell death can be caspase-dependent or caspase-independent. In the present study, cotreatment of cells with the caspase inhibitor Z-VAD-FMK, which abrogated GSE-induced activation of caspases and apoptosis, has failed to prevent JNK activation. Such findings indicate that activation of JNK by GSE does not represent a secondary, caspase-dependent event. It was also noted that inhibition of JNK activation by either a specific JNK inhibitor, SP600125, or JNK siRNA blocks activation of caspases and apoptosis. Furthermore, enforced activation of JNK significantly enhanced GSE-induced caspase activation and apoptosis. These data suggest that activation of JNK operates the upstream of caspase activation. This stress pathway plays a critical functional role in apoptosis induction by GSE.

Our present study has revealed that GSE causes strong up-regulation of Cip/p21 expression in human leukemia cells.

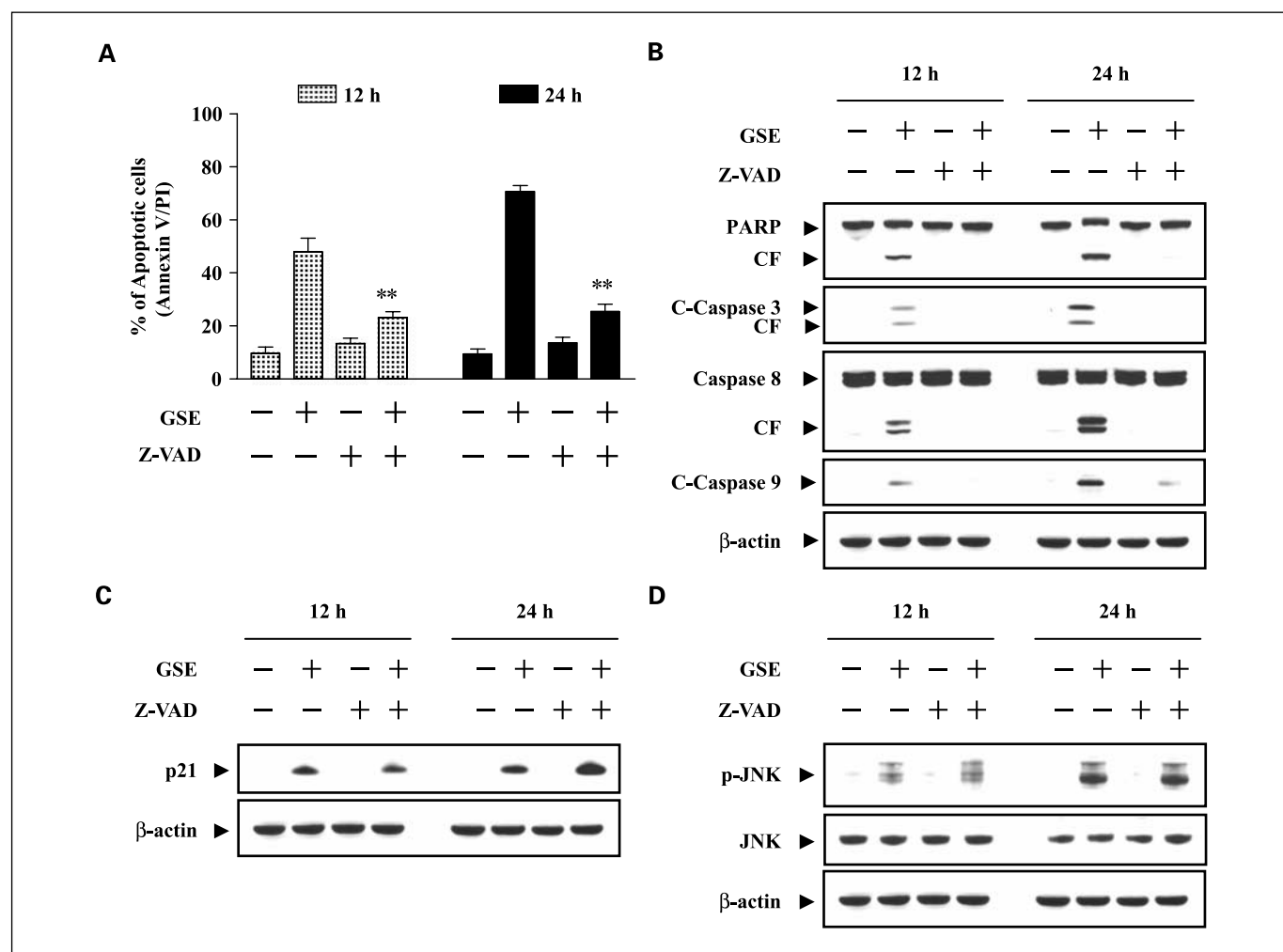


Fig. 5. Effects of inhibition of caspases by Z-VAD-FMK on apoptosis, activation of caspase, expression of Cip1/p21, and phosphorylation of JNK. Jurkat cells were pretreated with the caspase inhibitor Z-VAD-FMK (10 μ mol/L) for 1 h followed by treatment with 50 μ g/mL GSE for 24 h. *A*, cells were stained with Annexin V/PI. Apoptosis was determined using flow cytometry as described in Materials and Methods. Mean \pm SD of three separate experiments (obtained from Annexin V assays). **, $P < 0.01$, values for cells treated with both GSE and Z-VAD-FMK were significantly reduced compared with values obtained by GSE alone (Student's *t* test). *B* to *D*, total protein extracts were prepared and subjected to Western blot assay using antibodies against PARP, cleaved caspase-3, caspase-8, cleaved caspase-9, Cip1/p21, phospho-JNK, and JNK. For Western blot analysis, each lane was loaded with 30 μ g protein. Blots were subsequently stripped and reprobbed with antibody against β -actin to ensure equivalent loading. Two additional studies yielded equivalent results.

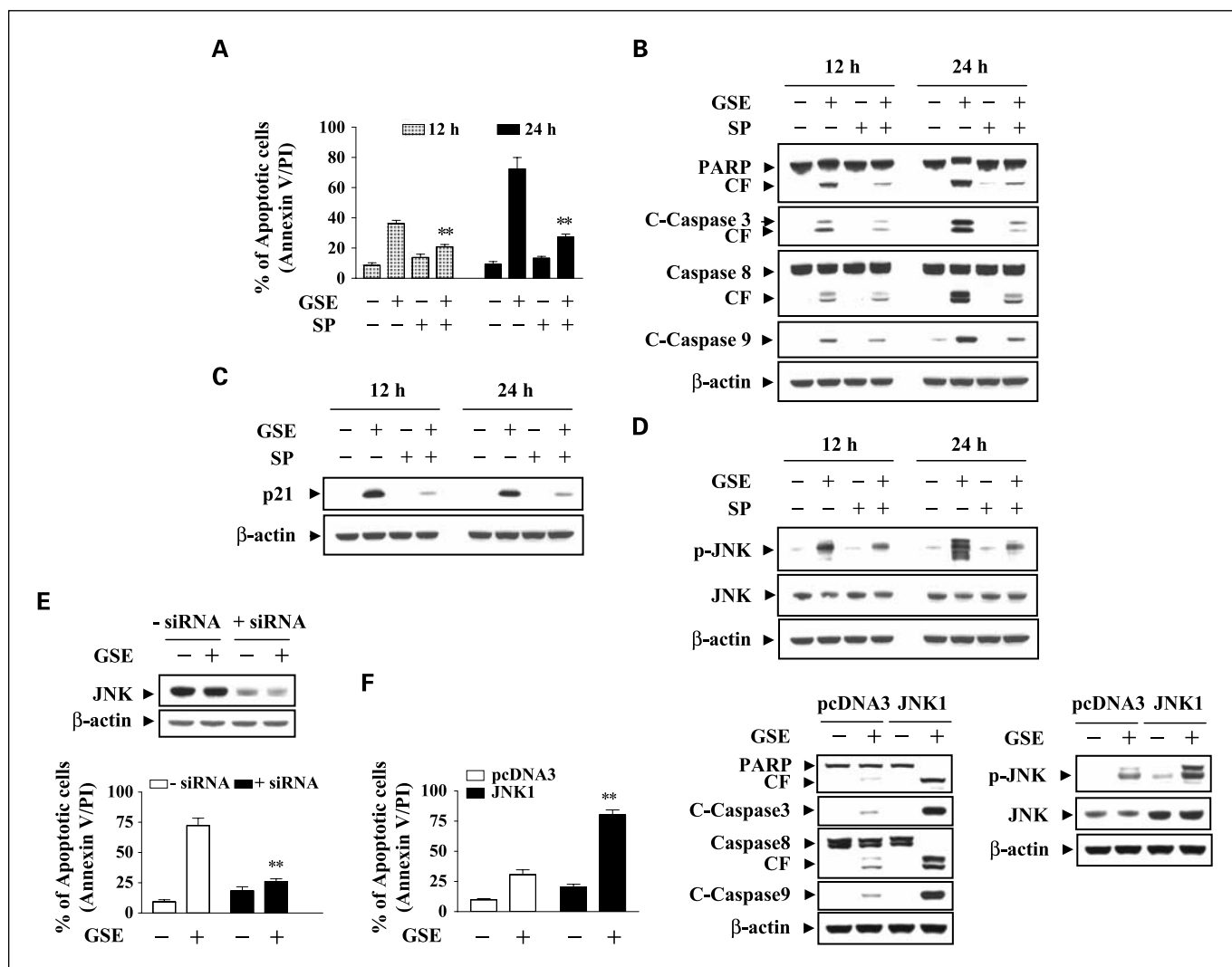


Fig. 6. Effects of pharmacologic and genetic inhibition of JNK and enforced activation of JNK on GSE-induced apoptosis. Jurkat cells were pretreated with 10 μ mol/L JNK inhibitor, SP600125 (SP), for 1 h followed by the addition of 50 μ g/mL GSE for 24 h. **A**, cells were stained with Annexin V/PI, and apoptosis was determined using flow cytometry as described in Materials and Methods. Mean \pm SD of three separate experiments (obtained from Annexin V/PI assays). **, $P < 0.01$, values for cells treated with both GSE and SP were significantly less than those obtained for cells treated with GSE alone (Student's t test). **B** to **D**, after treatment, total protein extracts were prepared and subjected to Western blot assay using antibodies against PARP, cleaved caspase-3, caspase-8, cleaved caspase-9, Cip1/p21, phospho-JNK, and JNK. **E**, Jurkat cells were transfected with JNK1 siRNA oligonucleotides or controls and incubated for 24 h at 37°C, after which cells were treated with 50 μ g/mL GSE for 24 h. Apoptosis was determined using the Annexin V/PI assay as described in Materials and Methods. **, $P < 0.01$, values for cells treated with GSE after transfection with JNK1 siRNA oligonucleotides were significantly decreased compared with those for control cells treated with GSE (Student's t test). Total cellular extracts were prepared and subjected to Western blot analysis using antibodies against JNK1. **F**, Jurkat cells were stably transfected with epitope-tagged JNK1 or an empty vector (pcDNA3) as described in Materials and Methods. JNK1 and pcDNA3 cells were treated with 10 μ g/mL GSE for 24 h, after which apoptosis was analyzed using Annexin V/PI assay. **, $P < 0.01$, values for JNK1 cells treated with GSE were significantly increased compared with those for pcDNA3 cells (Student's t test). Total cellular extracts were prepared and subjected to Western blot analysis using antibodies against PARP, cleaved caspase-3, caspase-8, cleaved caspase-9, phospho-JNK, and JNK. For Western blot assay, each lane was loaded with 30 μ g protein. Blots were subsequently stripped and reprobbed with antibody against β -actin to ensure equivalent loading. Two additional studies yielded equivalent results.

p21 protein is an inhibitor of cyclin-dependent kinase and plays an important role in regulating cyclin-dependent kinase activity and cell cycle progression in response to a wide variety of stimuli (45). In addition to normal cell cycle progression, p21 has been postulated to participate in growth suppression and apoptosis through a p53-dependent or p53-independent pathway following stress, and induction of p21 may cause cell cycle arrest (46, 47). In a recent study, GSE has been shown to inhibit cell growth and induce G₁-phase cell cycle arrest and apoptosis in human colorectal cancer cells and modulate cell cycle regulators with a strong effect

for Cip1/p21 up-regulation (21). Consistent with this result, GSE-mediated apoptosis in Jurkat cells may be associated with Cip/p21 up-regulation and cell cycle arrest. Additional mechanistic studies, however, are required in the future to elucidate how Cip1/p21 plays a role in GSE-induced apoptosis in human leukemia cells.

In the present study, we provide evidence that GSE causes up-regulation of Cip1/p21 through the activation of JNK in human leukemia cells. A link between the activation of JNK and up-regulation of Cip1/p21 is provided by the fact that SP600125, a selective inhibitor of JNK, effectively inhibits

Cip1/p21 up-regulation induced by GSE. Similar results are provided by a study in which galectin-8 induces cell cycle arrest and apoptosis through up-regulation of Cip1/p21 by activation of JNK (48). Inhibition of JNK activation by a selective inhibitor of JNK, SP600125, completely inhibits the up-regulation of Cip1/p21 mediated by galectin-8, suggesting that JNK seems to play the major role in the mechanism underlying the up-regulation of Cip1/p21. Another evidence supports a model in which transcription of Cip1/p21 gene is activated by early growth response-1 independently of p53 in response to curcumin treatment in U-87MG human glioma cells. Early growth response-1 expression is induced by curcumin through activation of JNK, suggesting that JNK/early growth response-1 signal cascade is required for p53-independent transcriptional activation of Cip1/p21 (49). Collectively, our observations suggest a hierarchy of events in GSE-induced lethality in which JNK activation represents

the early insult, which leads to Cip1/p21 up-regulation and caspase activation and apoptosis.

In summary, the present study has provided evidence that GSE induces human leukemia cell death with the activation of caspase-3, -8, and -9 as well as PARP cleavage and that GSE-induced apoptosis is preceded by the activation of JNK and thus up-regulation of Cip1/p21. The data presented here suggest that Cip1/p21 and JNK signaling pathway may represent attractive targets to GSE-induced apoptosis in human leukemia cells. The results of this study could have implications for the incorporation of agents such as GSE into the chemopreventive or therapeutic intervention against leukemia and possibly other hematologic malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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