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# Methylglyoxal Induces Apoptosis in Jurkat Leukemia T Cells by Activating c-Jun N-terminal Kinase

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**Abstract** Methylglyoxal (MG) is a physiological metabolite, but it is known to be toxic, inducing stress in cells and causing apoptosis. This study examines molecular mechanisms in the MG-induced signal transduction leading to apoptosis, focusing particularly on the role of JNK activation. We first confirmed that MG caused apoptosis in Jurkat cells and that it was cell type dependent because it failed to induce apoptosis in MOLT-4, HeLa, or COS-7 cells. A caspase inhibitor, Z-DEVD-fmk, completely blocked MG-induced poly(ADP-ribose)polymerase (PARP) cleavage and apoptosis, showing the critical role of caspase activation. Inhibition of JNK activity by a JNK inhibitor, curcumin, remarkably reduced MG-induced caspase-3 activation, PARP cleavage, and apoptosis. Stable expression of the dominant negative mutant of JNK also protected cells against apoptosis notably, although not completely. Correspondingly, loss of the mitochondrial membrane potential induced by MG was decreased by the dominant negative JNK. These results confirmed a crucial role of JNK working upstream of caspases, as well as an involvement of JNK in affecting the mitochondrial membrane potential. J. Cell. Biochem. 77:333–344, 2000. 

**Key words:** methylglyoxal; apoptosis; JNK; caspase; Jurkat

Methylglyoxal (MG) is a reactive α-oxoaldehyde and a physiological metabolite produced by various metabolic pathways, including the fragmentation of triose-phosphates or the metabolism of acetone and aminoacetone [Thornalley, 1993, 1996]. An increase in the concentration of MG in tissues and body fluids is strongly related to the development of diabetes complications such as retinopathy, neuropathy, and nephropathy [Mclellan et al., 1994]. It is known that MG, albeit a natural biological product, is toxic and induces stress in cells. When cells are exposed to various kinds of physical or chemical stress, this stress causes multiple events in cellular components involved in the intracellular signal transduction pathways, resulting in cellular proliferation, development, or death. Thus, apoptosis, a programmed cell death, is one of the usual results of stress; some studies have proved that MG induces apoptosis in some cell lines, including HL-60 [Kang et al., 1996] and U937 [Okado et al., 1996]. Although the action of MG in influencing cellular components has been studied, and amino acid residues affected by MG have been identified, detailed molecular events caused by MG, which activates the intracellular signal transduction pathway and leads the cells to apoptosis, have not yet been clarified.

Activation of caspases and the subsequent cleavage of specific proteins are the irreversible gates for apoptosis in most cells [Nancy et al., 1998; Allen et al., 1998; Cryns and Yuan, 1998]. Possibly, caspases could be activated by two interacting and reversible pathways: the mitochondrial route and the death receptor route. In the former, cytochrome c, released from mitochondria into the cytoplasm, binds to

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Apaf-1, the mammalian homologue of CED-4, which in turn interacts with procaspase-9 to form an apoptosome, resulting in the release of mature caspase-9 [Saleh et al., 1999; Zou et al., 1999]. The mature caspase-9 proteolytically activates the downstream caspases such as caspase-3. In the latter route, binding of death ligands such as tumor necrosis factor-α (TNF-α) or FasL to their receptors triggers the recruitment of the most upstream procaspases (procaspase-8 and -10) to the site of cytoplasmic death domain (DD) in the receptor molecules, causing proteolytic activation of these caspases [Schulze et al., 1998]. Whichever the activating route of caspases, the activated downstream caspases instigate and initiate a proteolytic cascade, resulting in the ultimate demise of the cell.

JNK (c-Jun N-terminal kinase) is involved in the intracellular signal transduction pathways in response to a wide variety of different stimuli, and control cell growth, differentiation, and stress responses [Cowley et al., 1994; Kyriakis et al., 1994; Gupta et al., 1995]. During the process of apoptotic signal transmission, strong activation of JNK is often observed, suggesting a relevance between JNK signal pathways and apoptosis [Verheij et al., 1996; Xia et al., 1995; Johnson et al., 1996; Huang et al., 1997]. One of the major issues regarding the signal pathway and JNK is where and how the latter is involved in the two specific routes of apoptosis described above. Although many reports have been issued on this problem and investigated using different cell types and apoptosis-inducing reagents, some controversial results have been described regarding the position of JNK in the signal pathway leading to apoptosis. Based on the order of JNK activation and caspase(s) activation, the reports may be divided into two contrastive descriptions, i.e., whether JNK activation is upstream or downstream of caspase(s) in the apoptosis-inducing signal transduction.

Although it is still impossible to conclude from the results of these reports, the order of activation of JNK and caspase(s) seems to depend mainly on the stimulation type. When cells are stimulated by the death receptors such as Fas and TNF-receptor, JNK activation, even if it is observed, usually takes place after caspase activation [Cahill et al., 1996; Juo et al., 1998; Chaudhary et al., 1999]. In this case, activation of JNK may not be necessary for the apoptotic process because inhibition of the JNK pathway by expressing dominant-negative mutant forms of MEKK1, SEK1, or c-Jun does not rescue cells from Fas- or TNF-mediated cell death [Wilson et al., 1999; Liu et al., 1996; Lenczowsdo et al., 1997; Low et al., 1999]. By contrast, JNK activation precedes caspases when the cells are exposed to stresses, including many chemical agents, irradiation, and oxidation [Zhang et al., 1999; Hashimoto et al., 1999; Wang et al., 1999; Inanami et al., 1999; Turner et al., 1998; Chen et al., 1998; Harada and Sugimoto 1999; Seimiya et al., 1997]. In this case, however, how and where JNK interacts with the target molecule(s) and participates in the signal transduction pathway remain unidentified. As a consequence, the roles of JNK in apoptosis remain debatable.

In the present study, using a T-cell line Jurkat, we have examined the signal transduction and target molecules in the apoptotic process induced by a potent stress-inducer MG. Our initial attempt in this study was to confirm the induction of apoptosis by MG and to examine cell type selectivity in MG-induced apoptosis. We then examined the molecular mechanisms in MG-induced apoptosis, especially in the role of JNK for signal transduction, leading to apoptosis. Our results indicate that MG selectively induces apoptosis in Jurkat cells, where the activation of JNK, which possibly affects the mitochondrial membrane potential, is an important process in the signal pathway to apoptosis.

## MATERIALS AND METHODS

### Antibodies and Chemical Reagents

Anti-phospho-JNK (threonine 183/tyrosine 185) and anti-phospho-c-Jun (serine 73) antibodies were purchased from New England Biolabs (Beverly, MA); anti-JNK, anti-CPP32 and anti-human PARP antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Methylglyoxal, curcumin, and 3,3-dihexyloxacarbocyanine iodide (DiOC6) were purchased from Sigma Chemical Co. (St. Louis, MO), the annexin V assay kit from Trevigen (Gaithersburg, MD), and Z-DEVD-fmk from Medical & Biological Laboratories (Nagoya, Japan).

### Cell Culture

Jurkat cells (human T-cell leukemia), MOLT-4 cells (human T-cell leukemia), and HeLa cells (human cervical adenocarcinoma) were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FCS) and 50 μM β-mercaptoethanol. COS-7 cells (transformed monkey kidney fibroblasts) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. One day before the experiment, cell density was adjusted to $5 \times 10^5$/ml, and cells were cultured in a fresh medium without β-mercaptoethanol.

### Flow Cytometric Analysis of DNA Fragmentation

Jurkat cells were resuspended at $2.5 \times 10^5$ cells/ml in RPMI 1640 medium supplemented with 5% FCS and plated into each well of a 96-well tissue culture plate ($5 \times 10^4$ cells/well). After stimulation, cells were harvested, resuspended in 500 μl of propidium iodide buffer (0.1% Triton X-100, 0.1% trisodium citrate) containing 50 μg/ml propidium iodide, and incubated for 15 min on ice. DNA fragmentation analysis was carried out using a FACScalibur flow cytometer (Becton Dickinson), as described previously [Ohkusu et al., 1997]. Apoptosis was scored by the percentage of cells appearing in the area below the G1/G0 peak.

### Annexin V-Binding Analysis

In this study, $2 \times 10^5$ cells were plated into a 24-well plate in the medium as above. After treatment with MG, cells were harvested at the indicated time, washed with phosphate-buffered saline (PBS), and resuspended in annexin V-binding buffer. FITC-conjugated annexin V was added to a final concentration of 100 ng/ml; cells were incubated in the dark for 15 min at room temperature. A total of 50 μg/ml of propidium iodide was added to each sample before the flow cytometric analysis.

### Assessment of Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured by 3,3′-dihexyloxacarbocyanine iodide (DiOC6) staining. $2 \times 10^5$ cells were treated with or without 0.25 mM MG, washed three times with PBS, and stained with 40 nM of DiOC6 at 37°C for 15 min. Control experiments with the complete abolishment of the mitochondrial membrane potential were performed by incubating cells with 5 μM of an uncoupling reagent, carbamoyl cyanide m-chlorophenylhydrazone (mClCCP), for 15 min at 37°C. Cells were analyzed by flow cytometry with an excitation light of 488 nm and an emission light of 525 nm.

### Cell Lysis and Immunoblot Analysis

The immunoblot assays were performed as described previously [Akhand et al., 1997]. Briefly, cells were lysed in lysis buffer containing 0.5% Nonidet P-40 (NP-40), 20 mM Tris-HCl (pH 7.6), 0.15 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium vanadate, 20 μg/ml aprotinin, and 5 μg/ml leupeptin. The lysates were cleared by centrifugation at 13,000*g* for 10 min, denatured by boiling in Laemmli buffer for 3 min, separated on 10% or 14% sodium dodecyl sulfate (SDS) polyacrylamide gels, and blotted onto nitrocellulose membrane. Nonspecific binding was blocked by incubating the membrane with 0.05% Tween-20/PBS containing 5% nonfat dry milk for 1 h at room temperature. Membranes were incubated with the primary antibody overnight at 4°C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody, and the specific immune complexes were detected using the Western blot plus Chemiluminescence Reagent (Life Science, Boston, MA).

### Plasmid Construction and Gene Transfection

pcDL-SR-JNK-VPF plasmid to express dominant negative JNK kinase was a generous gift from Dr. E. Nishida (Kyoto University), and pEGFP-C3 was purchased from Clontech (Palo Alto, CA). A part of pcDL-SR-JNK-VPF plasmid, containing a sufficient fragment to express HA-tagged JNK-VPF, was inserted at the multiple cloning site in pEGFP-C3 vector and disrupted the original GFP gene in order to avoid the expression of GFP product. The linearized plasmid was transfected into Jurkat cells by electroporation; $1 \times 10^7$ cells were suspended in PBS containing 40 μg of the plasmid DNA and pulsed at the condition of 270 V and 960 μF using a GenePulserII electroporator (Bio-Rad, Hercules, CA). After 48 h, cells were replated into a 96-well plate at $10^2$–$10^5$ cells/well and selected with 2 mg/ml of G418. Cells

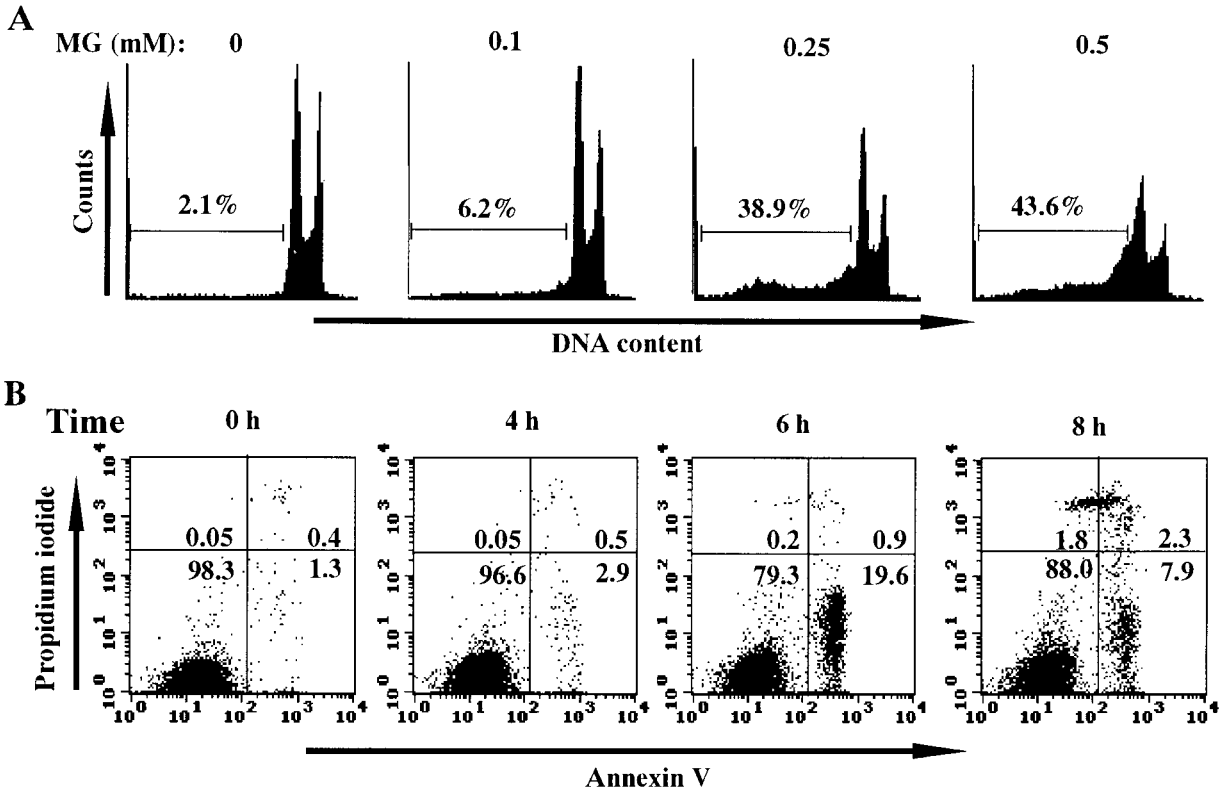


**Fig. 1.** Flow cytometric analysis of apoptosis induced by MG in Jurkat cells. **A:** Analysis of DNA fragmentation in MG-stimulated cells. Jurkat cells were incubated with the indicated concentration of MG for 24 h and analyzed by staining with propidium iodide. **B:** Annexin V binding and propidium iodide uptake of MG-stimulated Jurkat cells. Jurkat cells were incubated with 0.25 mM MG for 0–8 h at 37°C, and the percentage of apoptosis cells was assayed by flow cytometry. At least three separate experiments were carried out with results similar to the representative data shown here.

were also transfected with empty pEGFP-C3 vector alone to make control cells. After selection with the antibiotic for 14 days, resistant colonies were picked up and expanded. Three clones expressing dominant negative JNK were confirmed by immunoblot analysis with anti-HA and anti-JNK antibodies. Expression levels of dominant negative JNK protein were equal in these three clones and approximately 5 times as much as endogenous JNK levels. The clones were maintained in RPMI 1640 supplemented with 10% FCS containing 0.2 mg/ml of G418.

## RESULTS

### MG Induces Apoptosis in Jurkat Cells

We first asked whether MG caused apoptosis in cells of T-cell lineage. Human T-cell leukemia, Jurkat cells were treated with different concentrations of MG for 24 h, and their DNA fragmentation was determined by flow cytometric analysis, using propidium iodide as an indicator. It was clearly shown that MG induced cell death in a dose-dependent manner (Fig. 1A). The addition of 0.25 or 0.5 mM of MG in the culture media led a significant percentage of Jurkat cells to the area corresponding to a DNA content below 2N, which is a typical characteristic of apoptotic cells, together with a shrunken appearance. When Jurkat cells were treated with >0.5 mM of MG, however, cells died of necrosis without exhibiting DNA fragmentation, indicating the physical toxicity of a high concentration of MG (data not shown). In contrast to the action on Jurkat cells, MG did not affect MOLT-4, HeLa, or COS-7 cells for inducing DNA fragmentation in them (Table I).

After initiation of apoptosis, most types of cells translocate phosphatidylserine from the inner surface of the plasma membrane to the outside [Vermes et al., 1995], which is an early symptom of apoptosis and can be detected by annexin-V, a phospholipid-binding protein [Zhang et al., 1997; Koopman et al., 1994]. Fig-

**TABLE I. Effect of MG on Apoptosis in Different Cell Lines***

| Cell type | Conc. of MG (mM) | | |
|---|---|---|---|
| | 0 | 0.25 | 0.5 |
| Jurkat | 3.3 ± 2.0 | 38.5 ± 3.9 | 43 ± 2.5 |
| MOLT-4 | 4.8 ± 1.4 | 6.1 ± 1.9 | 5.8 ± 0.9 |
| HeLa | 4.6 ± 1.5 | 5.2 ± 1.0 | 6.6 ± 2.7 |
| COS-7 | 3.4 ± 1.5 | 3.7 ± 2.2 | 3.1 ± 2.0 |

*Four cell lines were treated with indicated concentration of MG for 24 h at 37°C. DNA fragmentation (mean ±SD; n = 4) was quantitated by propidium iodide staining and flow cytometry.

ure 1B shows that phosphatidylserine externalization in Jurkat cells was evident after 4 h of 0.25 mM MG treatment. Percentages of annexin-V-positive cells, most of which were negative for propidium iodide, further increased after 6 h of treatment. When exposed for 8 h, some of the cells stained with annexin-V turned positive for propidium iodide. After exposure to MG for more than 12 h, the percentage of cells positive for propidium iodide gradually increased (data not shown). This result indicated that the process of MG-induced apoptosis started within a couple of hours of MG treatment.

### Caspase-3 Protease Pathway Is Essential for MG-Induced Apoptosis in Jurkat Cells

Activation of caspase-3 is essential for the induction of apoptosis in many types of cells including Jurkat cells [Cohen, 1997; Villa et al., 1997]. Therefore, it is likely that MG induces apoptosis in Jurkat cells through activation of caspase-3. To test this possibility, we first determined whether MG was able to stimulate caspase-3 activity. Jurkat cells were treated with 0.25 mM of MG, and the activity of caspase-3 in cell extracts was measured by immunoblotting with an anti-caspase-3 antibody, which could also recognize activated forms of caspase-3, 17-kDa (p17) and 20-kDa (p20). Upon stimulation with MG, activation of caspase-3 with an appearance of p20 and p17 forms was observed at 2 h and 4 h (Fig. 2A).

During the process of apoptosis, PARP is inactivated due to specific cleavage of the protein into two fragments. The active form of caspase-3 is responsible for the cleavage of PARP through the recognition of a specific site. Thus, it was of interest to examine whether

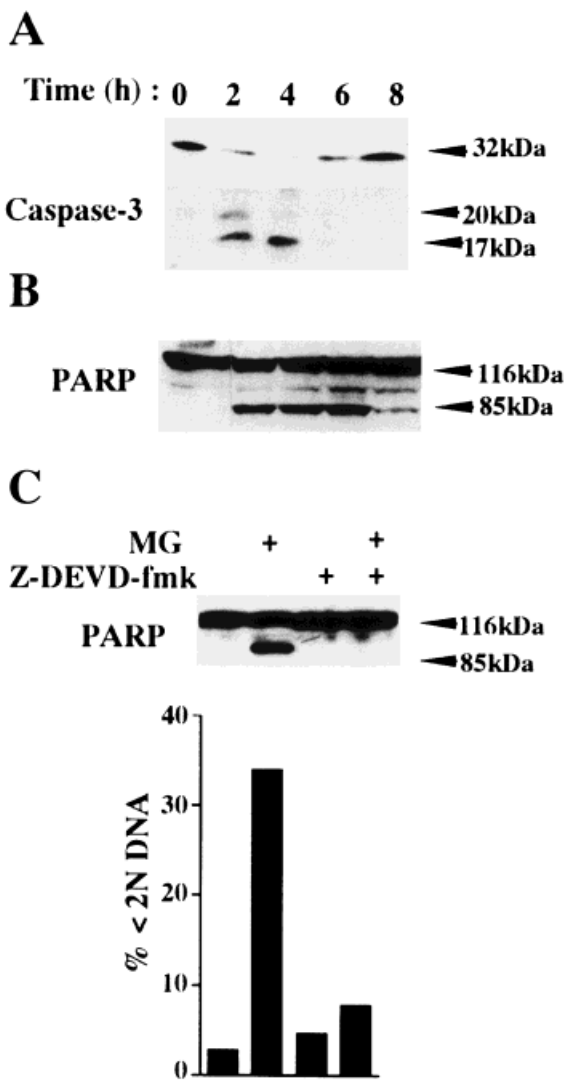


**Fig. 2.** Action of caspase-3 and PARP cleavage involved in MG-induced apoptosis in Jurkat cells. Jurkat cells were stimulated with 0.25 mM MG for indicated time, then caspase-3 (**A**) and PARP (**B**) were detected by immunoblotting analysis. Positions of the intact and cleaved forms are indicated by arrows. Cells were pre-incubated with or without 20 μM Z-DEVD-fmk for 24 h and treated with MG for 6 h for PARP cleavage analysis by immunoblotting, treated with 0.25 mM of MG for 24 h, followed by staining with propidium iodide and the flow cytometric analysis (**C**).

MG was also capable of inducing cleavage of PARP. As expected, MG induced an appearance of the inactivated 85-kDa form, which was evident at 2 h and maintained until 8 h after starting stimulation (Fig. 2B).

To determine more directly whether caspase protease was necessary for apoptosis to occur, a specific caspase-3 inhibitor, Z-DEVD-fmk, was used. Jurkat cells pretreated with the inhibitor

were allowed to undergo apoptosis by stimulation with MG, and the degree of apoptosis was determined by analysis measuring DNA contents. As shown in Fig. 2C, pretreatment with Z-DEVD-fmk completely blocked the MG-induced PARP cleavage and DNA fragmentation. These results indicated that caspase protease(s) were involved in MG-induced apoptosis process.

### MG Activates JNK in Jurkat Cells

Recent studies have demonstrated that multiple intracellular signaling pathways may lead to apoptosis; some of these pathways are being elucidated. In particular, some studies have suggested that JNK, a member of the MAPK superfamily, participates in the process leading to apoptosis induced by stress-inducing stimuli. Accordingly, we were interested to know whether MG was also able to activate these pathways. To test JNK phosphorylation, Jurkat cells were treated with different concentrations of MG for 1 h and were analyzed by immunoblotting with an anti-phospho-JNK antibody. Levels of phosphorylated JNK, which was undetectable in unstimulated cells, were significantly increased by treatment with 0.25 mM or 0.5 mM of MG in Jurkat cells (Fig. 3A).

To explore the relation between JNK phosphorylation and MG-induced apoptosis, we carried out a time-course study for JNK phosphorylation. Jurkat cells were treated with 0.25 mM of MG for 0–4 h, and activated JNK was examined by immunoblotting. Whereas the total level of JNK did not change, activated JNK significantly increased at 0.5, 1, and 2 h after initiation of stimulation (Fig. 3B).

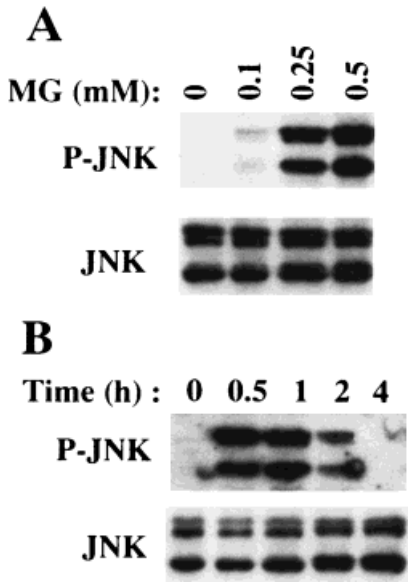


**Fig. 3.** JNK is activated in response to MG stimulation. **A:** Jurkat cells were treated with indicated concentration of MG for 1 h and then lysed in sample buffer after SDS-PAGE. The activated form of JNK was probed with anti-phospho-JNK antibody (top). The membranes were stripped of antibodies and immunoblotted with anti-JNK antibody (bottom). **B:** Jurkat cells were treated with 0.25 mM MG for the indicated time, and the activated form of JNK was probed as above.

### Interference With the JNK Pathway Suppresses MG-Induced Apoptosis and Downregulates Activation of Caspase-3

Curcumin works as a potent inhibitor of JNK activation by interfering with the molecule(s) upstream of JNK in the signal transduction pathway [Huang et al., 1994; Rao et al., 1995; Chen and Tan, 1998]. To confirm the role of the JNK pathway in MG-induced apoptosis, we preincubated Jurkat cells for 1 h with 20 μM of curcumin and treated them with 0.25 mM of MG. As shown in Figure 4A, activation of JNK, which is clearly induced by MG in cells without curcumin treatment, was completely inhibited by pretreating cells with curcumin. Because a functional cross-talk between JNK and the caspase signaling pathway has been reported, we tested the effect of curcumin on MG-induced activation of caspase-3. The result presented in Figure 4B showed that pretreatment with curcumin evidently decreased the formation of p17, an activated form of caspase-3. This result implied that JNK activation was an important step in Jurkat cells for the MG-induced signal transduction leading to the activation of caspase-3. Under this condition, we also observed that MG-induced PARP cleavage was notably reduced and DNA fragmentation induced by MG was partially suppressed by curcumin treatment, changing the percentage of cells with DNA content under 2N from 38% to 12% (Fig. 4C). These results indicated that the JNK phosphorylation was involved in MG-induced apoptosis.

### Suppression of JNK Activity by Dominant Negative Mutant JNK Inhibits MG-Induced Apoptosis

We looked for further evidence to characterize the functional role of JNK in MG-induced apoptosis by analyzing Jurkat cells transfected

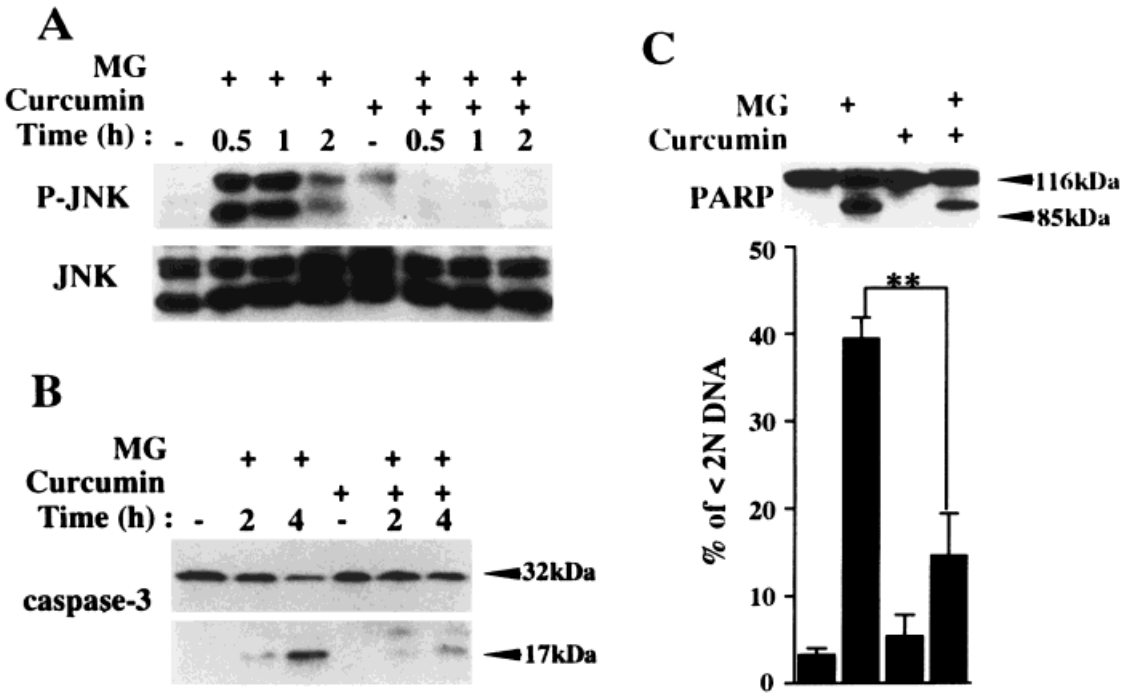


**Fig. 4.** Effects of curcumin on response to MG. Jurkat cells were preincubated with or without 20 μM curcumin for 2 h and treated with 0.25 mM MG for 1 h. JNK activity was detected by immunoblot analysis (**A**). Cells were treated with 0.25 mM MG for 0, 2 and 4 h, and an activated form of caspase-3 was detected by immunoblot analysis (**B**). Cells were treated with MG for 6 h for PARP cleavage analysis by immunoblotting, or treated with 0.25 mM of MG for 24 h for staining with propidium iodide, followed by flow cytometric analysis (**C**). Results of DNA content analysis are expressed as percentage of apoptotic cells (mean ±SD) of three independent experiments. **Significantly different ($P < 0.001$).

with a dominant negative mutant of JNK that inhibits the endogenous JNK signaling pathway. First, a Jurkat-derived cell line constantly expressing dominant negative JNK was examined for JNK activity. We examined whether endogenous JNK was inhibited by transfection of dominant negative JNK by investigating the phosphorylation state of intracellular c-Jun, which indicates the activity of JNK. Cells that constantly express dominant negative JNK were treated with MG and subjected to immunoblot analysis with an anti-phospho-c-Jun antibody. The result showed that 0.25 mM MG induced phosphorylation of c-Jun in control cells after 1–2 h of treatment but failed to induce phosphorylation of c-Jun in cells expressing dominant negative JNK (Fig. 5A).

Next we examined the effect of expressing dominant negative JNK protein in Jurkat cells on MG-induced apoptosis. MG-induced activation of caspase-3 was notably diminished in these dominant negative JNK-expressing Jurkat cells compared with mock-transfected cells (Fig. 5B). After 6-h treatment with 0.25 mM MG (Fig. 5C), the percentage of positive cells for annexin-V-staining remarkably increased (from 1.6% to 24.3%) in cells transfected with the empty vector. After 24-h treatment with MG at the same concentration, DNA fragmentation also increased remarkably (Fig. 5D). However, the increase in annexin-V-positive cells expressing dominant negative JNK was less than one-half of that transfected with the control vector, and the trend of DNA fragmentation was obviously suppressed compared with the control cells. These results indicated that the expression of dominant negative JNK could weaken the MG-induced apoptosis in Jurkat cells.

During the process of apoptosis, the mitochondrial transmembrane potential decreases. This results in opening of the permeability transition (PT) pore [Zamzami et al., 1995; Petit et al., 1995; Chen et al., 1998] which causes a local disruption of the outer mitochondrial membrane. As a consequence, the release of soluble intermembrane proteins, including cytochrome c, which contributes to the capacity to activate caspases and nucleases [Kantrow et

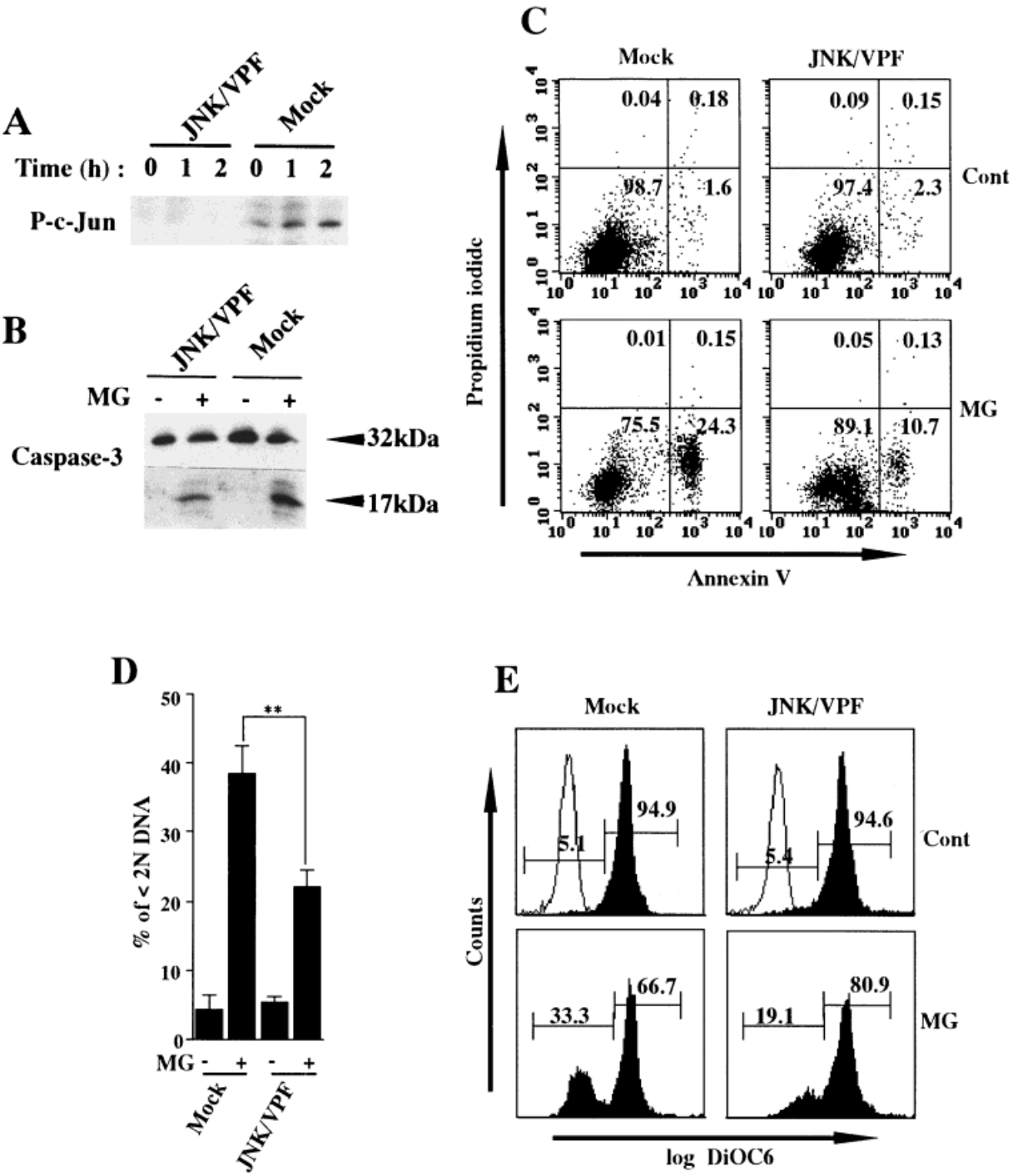


**Fig. 5.** Effect of expression of dominant negative JNK on response to MG stimulation in Jurkat cells. **A:** Jurkat cells expressing dominant negative JNK (JNK/VPF) or control vector (Mock) were treated with 0.25 mM MG for 0, 1, and 2 h, and cell lysates were subjected to Western blotting with anti-phospho-c-Jun antibody. **B:** Cells expressing JNK/VPF or empty vector (Mock) were treated with 0.25 mM MG for 4 h, and the activated form of caspase-3 (p17) was analyzed by immunoblot using anti-CPP32 antibody. **C:** Cells transfected with dominant negative JNK (JNK/VPF) or empty vector (Mock) were treated with 0.25 mM MG for 6 h, stained with FITC-conjugated annexin V, and propidium iodide, and analyzed by flow cytometry as described in the legend for Fig. 1B. **D:** These cells were treated with 0.25 mM MG for 24 h, and then DNA fragmentation was analyzed by flow cytometry with single propidium iodide staining. **Significantly different ($P < 0.001$). **E:** Alteration in mitochondrial membrane potential was measured by flow cytometry using DiOC6 staining. Cells transfected with JNK-VPF or empty vector (Mock) were treated with 0.25 mM MG for 2 h, and DiOC6 uptake was determined by flow cytometry as described under Materials and Methods. Control cells treated with mClCCP are overlaid in the upper histograms (open peak). All experiments were carried out at least three times; the results were similar to the representative data shown.

al., 1997; Petit et al., 1998]. Changes in the mitochondrial transmembrane potential can be detected by analyzing the incorporation of cationic lipophilic dye, DiOC6 [Kroemer et al., 1995, 1998]. Because the alteration in mitochondrial membrane potential is important in forming its membrane channels, we attempted to investigate whether the effect of JNK on a reduction in mitochondrial membrane potential is related to MG-induced apoptosis. The mitochondrial membrane potential of Jurkat cells expressing dominant negative JNK and of control cells (only transfected with the empty vector) were examined. During treatment with MG, an increase in the population losing mitochondrial membrane potential was observed in control cells, whereas the increase was less in cells expressing dominant negative JNK (Fig. 5E). These observations may suggest a mechanism whereby the activation of JNK may promote a reduction in mitochondrial membrane potential, followed by the process of apoptosis.

## DISCUSSION

In the present experiments, we observed that MG was sufficient to induce apoptosis in a dose-dependent manner in Jurkat cells. MG-induced cell death showed typical apoptotic features including DNA fragmentation and early externalization of phosphatidylserine. The finding that MG-induced cell death was completely blocked by Z-DEVD-fmk, a specific inhibitor of caspase-3, further confirmed that the cell death was caspase dependent. MG was reported to induce apoptosis in human leukemia HL-60 cells and in a macrophage-derived cell line U937 [Kang et al., 1996; Okado et al., 1996]. Our results agreed with the previous reports, further confirming an apoptosis-inducing potential of MG in another cell line. In contrast to the effect on Jurkat cells, however, MG showed no ability to induce apoptosis in MOLT-4 cells as another T-cell line, HeLa or COS-7 cells as non-T-cell lines. This suggests that the mechanism of MG-induced apoptosis is cell type-dependent.

We have shown that a strong activation of JNK was observed as early as 30 min after starting exposure to MG in Jurkat cells and was maintained for 2 h. Examination of apoptosis by the annexin-V staining method, which can detect the early stage of apoptosis, showed that apoptosis occurred as early as 4 h after initiation of MG treatment. These results suggested that activation of JNK preceded the onset of apoptosis, and possibly initiated an intracellular signaling event in preparation for apoptosis. In our experiment, both the inhibition of JNK activity by a JNK inhibitor (curcumin) and the suppression of JNK activation by expressing its dominant negative mutant, reduced MG-induced apoptosis. It appears that JNK activation is not required for the cell death mediated by death receptors such as Fas and TRAIL, whereas several lines of evidence suggest that JNK plays an important role in apoptosis induced by ultraviolet (UV) irradiation, oxidation, and other stress stimuli [Ip and Davis, 1998]. Our observations are consistent with these considerations and suggest that the apoptotic signal triggered by MG converges at a step downstream of JNK activation.

Recently, many reports have focused attention on the role of the JNK pathway, and have confirmed that activated JNK could be either upstream or downstream of the caspase activation of apoptotic cells. In the present study, phosphorylation of JNK increased as early as 30 min after initiation of MG stimulation; it apparently preceded the activation of caspase-3-like protease, which was not detected until 2 h after starting the MG stimulation. Thus, activation of JNK and of caspase-3-like protease probably occurs sequentially in apoptosis of Jurkat cells. Blockade of JNK by curcumin was also shown to reduce caspase activation and PARP cleavage, indicating that JNK is upstream of caspase-3 in the MG-induced pathway. Our results agree with the previously described ones using other stress-inducing reagents [Turner et al., 1998; Chen et al., 1998]. Our observation that the inhibition of JNK cascade did not completely block caspase-3 activation and apoptosis may suggest that the role of JNK for MG-induced apoptosis could be partly compensated for by some other molecules, and that some dispensable pathways for apoptosis escaping JNK may exist. Activation of p38 kinase has been best characterized in response to stress-inducing stimuli such as UV light or hyperosmolarity, as well as in response to cytokines such as interleukin-1 (IL-1) or TNF [Xia et al., 1995; Han et al., 1994]. It is possible that p38 kinase also plays a role in MG-induced apoptosis. The nature of this kinase pathway and the relationship of interaction between JNK and p38 kinase in apoptosis remains to be defined.

A significant reduction in the incorporation of DiOC6 was observed after MG treatment. That reduction was suppressed by expressing the dominant negative mutant of JNK, which completely blocked the activation of endogenous JNK but partially inhibited the apoptosis (Fig. 5E). This result may indicate that JNK functions to promote the loss of mitochondrial transmembrane potential. It is generally known that members of the Bcl-2 family, including Bcl-2, Bcl-xL, and Bax, regulate mitochondrial membrane permeability [Marzo et al., 1998a,b]. Bcl-2 and Bcl-xL are shown to strengthen the mitochondrial membrane to be resistant to pore opening. By contrast, it was reported that JNK was able to phosphorylate and inactivate Bcl-2 [Maundrell et al., 1997; Ito et al., 1997; Rakesh et al., 1999]. Therefore, one of the possible mechanisms for the resistance induction of mitochondrial membrane permeability by activated JNK is that JNK affects the function of Bcl-2. Another possible mechanism is that JNK may activate downstream effectors of the stress pathways including the transcription factors c-Jun, ATP-2, and Elk-1, and that these transcription factors may, in turn, initiate the transcription of many proteins including Bax, which is able to bind to the permeability transition pore complex (PTPC), and thus to regulate mitochondrial membrane permeability [Marzo et al., 1998b]. It is also possible that JNK may affect the function of some unidentified protein(s) involved in the reduction of mitochondrial membrane permeability. Identification and verification of the possibilities raised above is the next subject to be investigated.

In summary, our work established that MG is capable of inducing apoptosis in a cell-type dependent manner, and that this apoptosis is accomplished by the induction of caspase-3 activation. JNK is crucial for this apoptosis to a considerable extent and is active upstream of caspases by affecting mitochondrial membrane potential, which is one possible cascade for apoptosis induction. This study is the first to propose that activation of JNK participates in MG-induced apoptosis, possibly by promoting the loss of mitochondria membrane potential. Further work may provide valuable insights into the molecular mechanisms and the role of JNK in apoptosis.

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