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# Inhibition of AP-1 Transcription Activator Induces Myc-Dependent Apoptosis in HL60 Cells

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**Abstract** Transcriptional activation of AP-1 is intricately involved in cell proliferation and transformation. The natural product, nordihydroguaiaretic acid (NDGA) shows an inhibitory effect on the binding of jun/AP-1 protein to the AP-1 site in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-stimulated HL60 cells. The NDGA inhibits the auto-regulated de novo synthesis of c-jun mRNA in TPA-stimulated HL60 cells. Our data also determine that this compound induces proliferation inhibition and apoptosis in human leukemia HL60 cells. To obtain information on the functional role of the AP-1 inhibition by NDGA in apoptosis signaling, the effects of pharmacological inhibition of AP-1 binding on c-myc, p53, and bax protein level were determined. Our results indicate that treatment of cells with NDGA enhances c-myc, p53, and bax protein levels. To rule out the possibility that NDGA will induce apoptosis because of the effects on proteins other than AP-1, we investigated the effect of another AP-1 inhibitor, SP600125, which is specific to Jun-N-terminal kinase. SP600125 decreased not only the phosphorylation level of jun protein but also AP-1/DNA binding activity. Also, apoptosis was observed to be induced by SP600125, concomitant with the increase in c-myc, p53, and bax protein level. In addition, apoptosis induced by both AP-1 inhibitors was accompanied by the activation of a downstream apoptotic cascade such as caspase 9, caspase 3, and poly[ADP-ribose]polymerase (PARP). When the cells were treated with NDGA or SP600125 in the presence of antisense c-myc oligonucleotides, apoptosis was not observed and an increase of c-myc, p53, and bax proteins was not manifested. All these results show that the inhibition of the transcription factor AP-1 action is related with either the drug-induced apoptosis or the drug toxicity of the HL60 cells. The apoptosis induced by AP-1 inhibition may be dependent on c-myc protein levels suggesting that the c-myc protein induces apoptosis at a low level of AP-1 binding activity. Altogether, our findings suggest that the presence of the AP-1 signal acts as a survival factor that determines the outcome of myc-induced proliferation or apoptosis. J. Cell. Biochem. 91: 973–986, 2004. 

**Key words:** jun/AP-1; inhibitor; HL60 cell; apoptosis; c-myc

Transcription factors play an important role in the expression of genetic information in the final stage of intracellular signaling. One such factor, activator protein 1 (AP-1), is a heterodimer formed by the products of the fos and jun, proto-oncogene families [Johnson and McKnight, 1989; Mitchell and Tjian, 1989]. The fos and jun proteins have almost identical amino acid sequences that comprise the basic DNA binding sequence (B) and the adjacent leucine zipper region (Zip), by which the proteins dimerize with each other [Johnson and McKnight, 1989; Mitchell and Tjian, 1989; Angel and Karin, 1991]. The AP-1 transcription factor binds specifically to the DNA sequence 5′-TGAG/CTCA, referred to as the AP-1 site and which is identical to the 12-*O*-tetradecanoylphorbol-13-acetate (TPA) responsive element (TRE) [Johnson and McKnight, 1989; Mitchell and Tjian, 1989]. The transcription of jun is stimulated by its own product. The c-jun may be

Abbreviations used: AP-1, activator protein-1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; NDGA, nordihydroguaiaretic acid; JNK, c-jun N-terminal kinase; $IC_{50}$, 50% inhibitory concentration; EMSA, electrophoresis mobility shift assay; FBS, fetal bovine serum; B-Zip, leucine zipper and basic domain; PKC, protein kinase C; PBS, phosphate-buffered saline; PARP, poly[ADP-ribose]polymerase; FRK, c-fos regulating kinase.

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permanently activated or over-expressed, which could lead to cell transformation [Schutte et al., 1988; Mitchell and Tjian, 1989; Angel and Karin, 1991]. Enhanced levels in the expression of c-jun and c-fos, as well as of AP-1-dependent genes, are found in tumors derived from in vivo and in vitro transformation [Schutte et al., 1989; Van der Burg et al., 1989]. In addition, c-jun is known to be over-expressed between 4- and 12-fold in 40% of human small-cell lung cancers and 20% of non-small cell lung cancers [Schutte et al., 1988]. Jun may be involved in leukemia [Mattei et al., 1990]. The activation of c-jun is probably crucial in transmitting cancer-promoting signals. The activation of protein kinase C (PKC) causes dephosphorylation of c-jun at the sites that negatively regulate its DNA-binding activity. TPA is known to activate PKC and, leads to the increased binding of the c-jun/AP-1 protein to the AP-1 site [Angel et al., 1987].

A variety of anti-cancer drugs have been shown to induce apoptosis in proliferating normal cells and tumor cells [Gunji et al., 1991; Cotter et al., 1992; Singh et al., 2001]. The way in which anti-cancer drugs induce apoptosis is not uniformly derived. However, many anti-cancer drugs mediate their therapeutic effect by triggering apoptosis [Kerr et al., 1994]. Existing data have been suggested to support the hypothesis that activation of apoptotic cell death in renal epithelial cells involves the induction of c-myc [Zhan et al., 1997]. There are some data to support the idea that c-myc expression contributes to apoptosis induced by cytokine and environmental stress [Dang et al., 1999; Kim et al., 2000; Pelengaris et al., 2000]. In addition, p53 is known to be required for c-myc-induced apoptosis of some cell types [Hermeking and Eick, 1994; Wagner et al., 1994; Jiang et al., 2001]. To understand the mechanisms by which AP-1 inhibitor induces apoptosis in cancer cells, the role of putative c-myc and p53 protein expression was analyzed. TPA-stimulated HL60 cells could be used as a model for c-jun over-expressed cancer. Recent studies from our laboratory have shown that the natural product, nordihydroguaiaretic acid (NDGA) suppressed binding of AP-1 and DNA in an in vitro screening assay [Park et al., 1998, 2000]. In this experiment, we performed tests to determine whether the natural product, NDGA suppresses AP-1 activation in TPA-stimulated HL60 cells using electrophoresis mobility shift assay (EMSA). Our findings show that NDGA suppresses AP-1 activation by interfering with the protein and DNA binding. There are also data indicating that NDGA inhibits proliferation and induces apoptosis in cancer cell line HL60. To rule out in this work that NDGA will induce apoptosis because of effects on proteins other than AP-1, Jun N-terminal kinase (JNK) inhibitor, SP600125, which results in inhibition of AP-1, was also used. These studies suggest that the AP-1 transcription activator may be an important target of cell growth inhibition. It has been reported that the SP600125 dose dependently inhibited the phosphorylation of c-jun, the expression of inflammatory genes *cox-2*, *IL-2*, *IFN-*$\gamma$, *TNF-*$\alpha$ in human cell cultures [Bennett et al., 2001; Han et al., 2001].

In the modified dual signal model previously studied, c-Myc coordinatedly induces proliferation and primes apoptosis through one pathway and triggers apoptosis through a second pathway. In this model, survival factors suppress the apoptosis trigger pathway [Prendergast, 1999]. In this report, we show that AP-1 inhibition leads to apoptosis in the HL60 cell through the induction of associated c-myc and p53. Thus, we suggest that the AP-1 activator acts as one of the survival factors blocking the apoptosis trigger pathway.

## MATERIALS AND METHODS

### Materials

The NDGA was purchased from Sigma Chemical Co. (St. Louis, MO) and the SP600125 from Biomol Research Laboratories, Inc., (Plymouth Meeting, PA).

### Cell Culture

HL60 human leukemia cell line was purchased from the Korean Cell Line Bank. The HL60 cells were grown in RPMI 1640 containing 10% fetal bovine serum and antibiotic-antimicotic solution (Gibco BRL, Rockville, MD).

### Preparation of Cell Extracts and EMSA

HL60 ($1 \times 10^7$) cells were lysed by incubation at 4°C for 10 min in 400 μl of buffer A (10 mM KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)). The cell lysate was centrifuged for 6 min, supernatant was stored at −70°C as a cytosolic extract until necessary after measurement of protein content, and the resulting pellet

was resuspended in 100 μl of ice-cold buffer C (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF). After incubation at 4°C for 20 min, the extract was centrifuged for 6 min, and supernatant was collected, aliquoted, and stored at −70°C as a nuclear extract [Dent and Latchman, 1993]. The protein content of the final extracts was estimated using the BCA kit according to the protocol of the manufacturer Bio-Rad (Richmond, CA). A labeled DNA probe was prepared by combining 1.7 pmol of AP-1 consensus oligonucleotide (purchased from Santa Cruz, CA) with 10 μCi [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase. The labeled DNA probe and cell extract were incubated for 30 min in buffer conditions of 20% glycerol, 5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 0.25 mg/ml poly(dI-dC) for the binding reaction [Sassone-Corsi et al., 1988]. Electrophoresis was performed on a pre-run 6% nondenaturing polyacrylamide gel (0.25× TBE, 130 V, 40 min). The band-shifts were detected by autoradiography.

### Northern Blot Analysis

Total cellular RNA was prepared using the Trizol isolation kit (Gibco BRL), separated by denaturing agarose gel, and transferred to the nitrocellulose membrane. Hybridization was performed for 16 h at 42°C in a hybridization buffer. A DNA probe of c-jun was prepared from an Eco*RI*–Hind*III* fragment of a 3′ c-jun b-Zip sequence [Glover and Harrison, 1995]. The cDNA probe was labeled with [$\alpha$-$^{32}$P]dCTP using a random primer extension kit (NEN, Boston, MA).

### c-Fos and c-jun B-ZIP Region Expression in *E. coli*

Cloning vectors (pTK) containing the c-fos and the c-jun B-Zip regions (histidine-tagged) were provided by T. Curran, St. Jude Children's Hospital. These vectors were expressed in *E. coli* BL21 containing the DE3 promoter by adding 1 mM IPTG and purified using a histidine-binding affinity column.

### Western Blot Analysis

HL60 ($5 \times 10^6$) cells were harvested and washed, and the pellet was suspended with 1.0 ml of ice-cold RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 ng/ml PMSF, 0.03% Aprotinin, 1 μM sodium orthovanadate), and incubated on ice for 30 min. After incubation on ice, the lysates were centrifuged and the supernatants were boiled in an SDS sample buffer. The proteins were separated on SDS–polyacrylamide gel electrophoresis (PAGE) and electroblotted to the nitrocellulose membrane. The proteins were detected by the ECL Western blotting analysis system (Amersham Pharmacia, Arlington Heights, IL) using anti-c-jun/AP-1 (D) antibody, anti p-c-jun (Ser63/73) antibody, anti c-myc antibody, p53 antibody, bax antibody, caspase 9 antibody, caspase 3 antibody, poly[ADP-ribose]polymerase (PARP) antibody, or GAPDH antibody (Santa Cruz), respectively. Results were normalized against GAPDH levels.

### $^3$[H]-Thymidine Uptake Cell Proliferation Assay

HL60 cells were cultured in 96-well flat bottom microtiter plates (Costar, Cambridge, MA) in 0.2 ml of RPMI 1640 containing antibiotics (100 U penicillin and 100 μg streptomycin) and 10% FBS. Cultures were incubated at 37°C with 5% $CO_2$ for 24 h and were pulsed with 1 μCi of [$^3$H] thymidine (sp act 2 Ci/mmol; New England Nuclear, Boston, MA) during the last 4 h of the culture period. Cultures were harvested and [$^3$H]-thymidine incorporation was determined by liquid scintillation counter. The statistical significance of the differences between treated and untreated samples was evaluated using a Student's test. The difference was judged to be statistically significant if $P < 0.05$.

### DNA Fragmentation Analysis

After the treatment of NDGA, the cells were washed with phosphate-buffered saline and incubated in lysis buffer (Tris-HCl/pH 7.4, 20 mM EDTA, 0.1% NP-40, 0.5 mg/ml proteinase K) at 4°C for 30 min. Then, the lysates were treated with 1 μg/ml RNase A at 40°C for 30 min. DNA was extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1), precipitated with 0.1 volume of 3 M sodium acetate and 2 volume of absolute ethanol at −70°C. The isolated DNA was dissolved in distilled water and analyzed in 1.8% agarose gel.

### Flow Cytometry

For early apoptosis detection, cells were harvested and stained with fluorescein-isothiocyanate (FITC)-labeled annexin V and propidium iodide (Roche Mannheim, Germany)

according to the manufacturer's manual, and analyzed with a four-color fluorescence flow cytometer (FACS, Becton Dickinson, Franklin Lakes, NJ).

### Human c-myc Antisense Oligonucleotide and RT-PCR Anaysis

Two different oligonucleotides were synthesized from Genset Biotech Ltd. (San Diego, CA). An oligomer, antisense, whose sequence was complementary to the first five codons (starting from the initiation codon) of human c-myc mRNA [Heikkila et al., 1987]: 5′ aacgttgaggggcat 3′, and a second oligomer as a control, whose sequence is the same but in the opposite direction to the sequence of antisense oligomer [Capeans et al., 1998]: 5′ tacggggagttgcaa 3′. To examine whether the treatment of antisense c-myc oligonucleotide blocks endogenous c-myc expression, we performed RT-PCR. After total cellular RNA was prepared using the Trizol isolation kit (Gibco BRL), the entire population of mRNA molecules is converted into cDNA by priming with oligo-dT primers. Then, myc gene-specific PCR primers are added for amplification.

### Statistical Analysis

Each data point represents the mean of three individual values and standard deviations. The MINITAB program was used for ANOVA.

## RESULTS

### Suppression of Binding Activity Between jun/AP-1 and DNA by NDGA in TPA-Stimulated HL60 Cells

We first determined whether NDGA suppresses the jun/AP-1 and DNA binding activity in TPA-stimulated HL60 cells. Nuclear extracts were isolated and mixed with an AP-1 oligonucleotide after TPA treatment for 30 min, with or without NDGA. To investigate the formation of dimer and DNA complex, we used an EMSA. The band of interest, attributed to c-Jun-AP-1 protein, was defined, because this band was weakened when the cell extract was pre-incubated with anti-c-Jun/AP-1 antibody. NDGA-treatment inhibited the binding of c-jun/AP-1 protein with DNA, which was stimulated by the treatment of TPA (Fig. 1A). The immediate induction of AP-1 by TPA was resistant to cycloheximide, which is a protein synthesis inhibitor (Fig. 1A). This result indicates that the increased AP-1 activity at 30 min after TPA induction was via translational modification rather than via transcriptional protein synthesis. In addition, the inhibition by NDGA was detectable with or without cycloheximide (Fig. 1A). Thus, it may be concluded that such suppression by NDGA also is not via transcriptional protein synthesis but via post-translational modification. To further assess the inhibitory mechanism of NDGA, the c-fos and the c-jun B-Zip regions were over-expressed in vitro and used for the EMSA assay instead of nuclear extracts. The dimer and DNA interaction was disturbed by the NDGA (Fig. 1B). This result indicates that the inhibition of AP-1 activity by NDGA is due to interfering with the direct interaction between dimer and DNA.

### Inhibition of c-jun mRNA Expression by NDGA in TPA-Stimulated HL60 Cell

To clarify whether the inhibitory effect of NDGA on the dimeric transcription factor and AP-1 sequence could affect the transcription of *c-jun* gene, which includes the AP-1 sequence by itself, the TPA-stimulated expression of c-jun mRNA in serum-starved HL60 cells was investigated using the Northern blotting method. The TPA-stimulated c-jun mRNA level peaked at 60 min. Thus, the following experiments to determine the effects of the inhibitor were performed using a 1 h-incubation. The NDGA at 60 and 200 μM reduced the level of c-jun mRNA to 36 and 9%, respectively, compared with the level of TPA-stimulated mRNA (Fig. 2). This result shows that the inhibitory activity of NDGA can affect c-jun/AP-1 activation at both the post-translational level and at the mRNA synthesis level. The blocking of binding between the transcription factor and the DNA by NDGA is likely to modulate auto-regulated c-jun expression.

### Effects of NDGA and SP600125 on c-jun Protein Level and Phosphorylation

Having demonstrated that immediate inhibition of both TPA-stimulated AP-1 activity and c-jun mRNA synthesis by NDGA in HL60 cells, we then examined the effect of NDGA on the c-jun protein levels and AP-1 binding activity after treatment for 6 h. Furthermore, we investigated the effect of JNK inhibitor on phosphorylation of c-jun protein and AP-1 binding activity in this cell line. The cells were treated with SP 600125 for 6 h and total lysates were

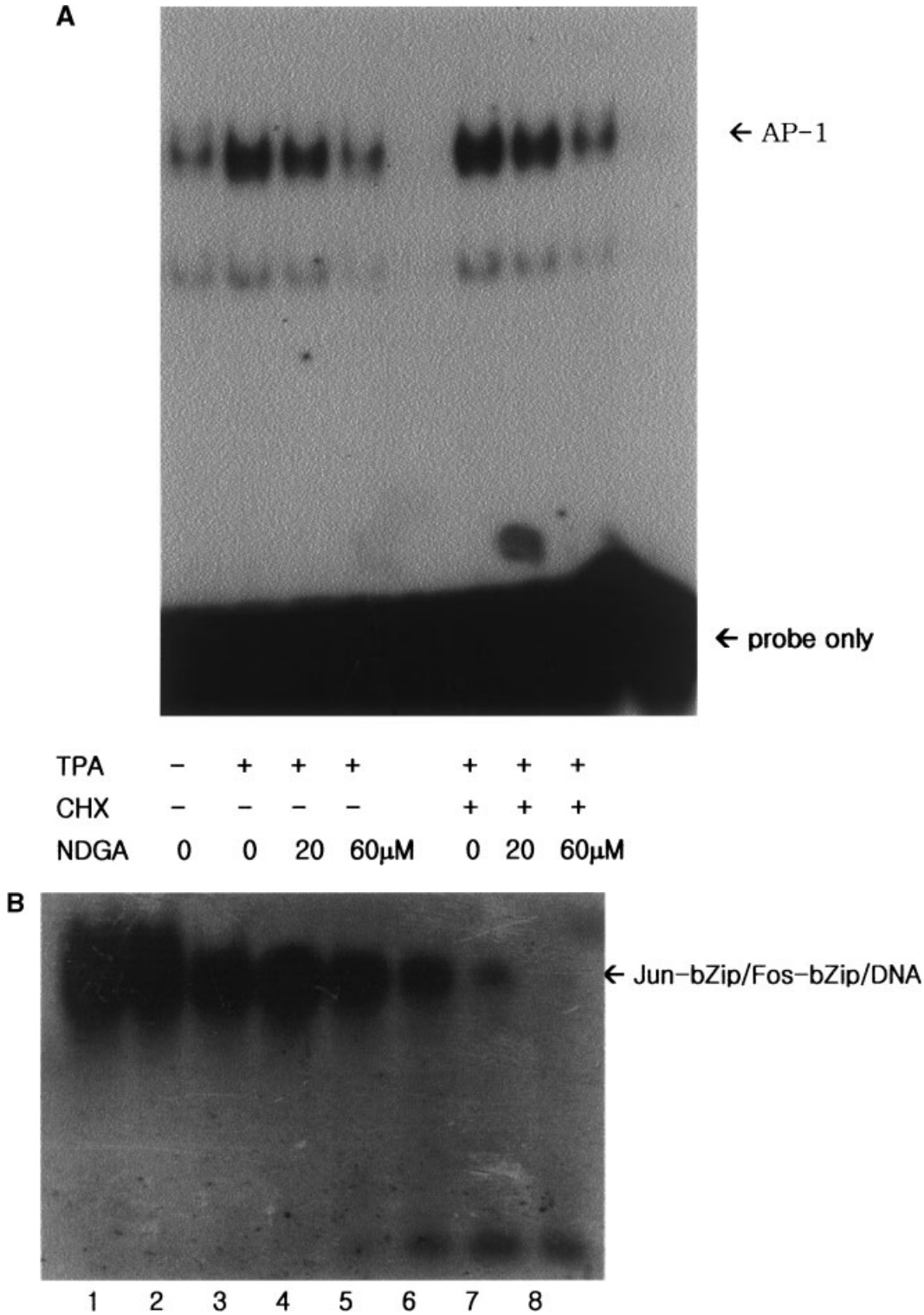


**Fig. 1.** **A**: Inhibition of AP-1 binding activity with the treatment of nordihydroguaiaretic acid (NDGA) in TPA-stimulated HL60 cells (EMSA). TPA and cycloheximide was used at concentrations of 50 ng/ml and 35 μM, respectively. Poly (dI-dC) (0.7 μg) were added. **B**: Autoradiography showing the effect of NDGA on fos–jun–DNA complex formation. Cloning vectors containing the c-fos and c-jun B-Zip regions were expressed in *E. coli* BL21 and dimerized proteins were used. Each protein containing 0.5 μM of jun and fos B-Zip regions was combined and 140 pM of radiolabeled AP-1 nucleotide was added. **Lane 1**, positive control (no inhibitor); **lane 2**, 0.25 μg of anti-c-Jun/AP-1 antibody; **lane 3**, 0.5 μg of anti-c-Jun/AP-1 antibody; **lane 4**, NDGA 20 μM; **lane 5**, NDGA 40 μM; **lane 6**, NDGA 60 μM; **lane 7**, NDGA 120 μM; **lane 8**, NDGA 200 μM.

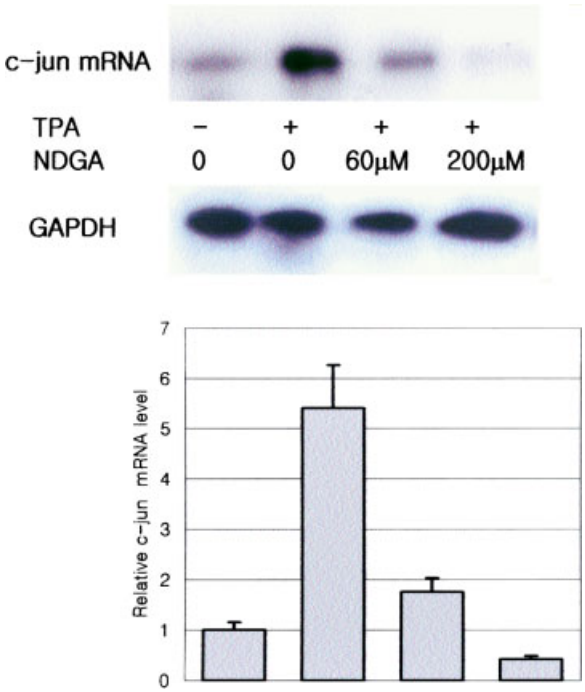


**Fig. 2.** Inhibition of c-jun mRNA expression with the treatment of NDGA in TPA-stimulated HL60 cell (Northern blotting). TPA was treated at 50 ng/ml for 1 h with or without an inhibitor. Densitometric analysis was performed on the autoradiograph. Signal intensities are expressed in arbitrary units. Each data point represents a mean of three individual values and standard deviations. The amount of c-jun mRNA was normalized with GAPDH mRNA. The MINITAB program was used for ANOVA followed by Dunnett's test. A statistically significant difference between NDGA-treated and -untreated samples ($*P < 0.01$) was observed. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

resolved by SDS–PAGE and immunoblotted with antibody against the phosphorylated form of c-jun. While NDGA blocked c-jun activity by decreasing the c-jun protein level, SP600125 blocked c-jun phosphorylation without an effect on the c-jun expression level (Fig. 3A–C). EMSA analysis showed that SP600125 lowered the AP-1 binding activity (Fig. 3D).

### Inhibition of HL60 Cell Proliferation by NDGA and SP600125

In subsequent studies, experiments were performed to determine the effects of pharmacological inhibition of AP-1 on the generation of growth inhibitory responses in HL60 cells. Cells were incubated for 24 h with inhibitor, and cell proliferation was determined by $^{3}$[H]-thymidine uptake assays. Treatment of HL60 cells with NDGA or SP600125 suppressed cell growth. As shown in Figure 4, the inhibition of cell proliferation depends on a dose of NDGA. The concentration of NDGA or SP600125 that kills 50% of the cells was determined to be 70 or 1.25 μM, respectively, when treated for 24 h.

### Apoptosis of HL60 Cells by the Inhibition of AP-1 by NDGA and SP600125

To investigate whether the cell death by NDGA or SP600125 was accompanied by apoptosis,apoptosis detection assays were performed. Treatment of HL60 cells with 70 μM NDGA or 1.25 μM SP600125 for 24 h induced DNA fragmentation, a hallmark of apoptosis (Fig. 5). To analyze the early phase apoptosis of cell population and to obtain quantitative data, annexin V-FITC/PI staining and, subsequent flow cytometry were performed. A four-color fluorescence flow cytometry (FACS analysis) allows the discrimination of intact cells (FITC−/PI−), apoptotic cells (FITC+/PI−), and necrotic cells (FITC+/PI+) [Vermes et al., 1995]. As shown in Figure 6, the treatment of 20 μM NDGA or 0.25 μM SP600125 for 24 h induced about 7.6 or 15.2% of early apoptotic cells, respectively. Also, it is observed that the number of apoptotic cells increased with the amount of treatment of NDGA. This finding, that AP-1 inhibition by either NDGA or SP600125 induces apoptosis, prompted us to perform further studies, to define whether such a process is related to known apoptotic intermediate proteins as c-myc, p53, and bax.

### Protein Level of c-myc, p53, bax, Caspase 9, Caspase 3 in HL60 Cells During Apoptosis by NDGA or SP600125

The protein level of c-myc, p53, and bax was studied in HL60 cells treated with AP-1 inhibitors using Western blot analysis. The results show that c-myc and p53 protein levels were increased from 6 h after the treatment of NDGA or SP600125 (Fig. 7A,B). The protein levels were quantified by densitometry and expressed as a relative level of controls (Fig. 7C,D). In addition to c-myc and p53, bax protein, which is an apoptotic intermediate of the intracellular mechanism is seen as a significantly strengthened band compared with that of the control ($^{bax}P = 0.047$) during the AP-1 inhibitor-induced responses of these cells.

Previous studies have demonstrated that cultured leukemia cells treated with chemotherapeutic agents undergo apoptosis via the activation of caspases [Soignet et al., 1998]. Also it is known that PARP is proteolytically

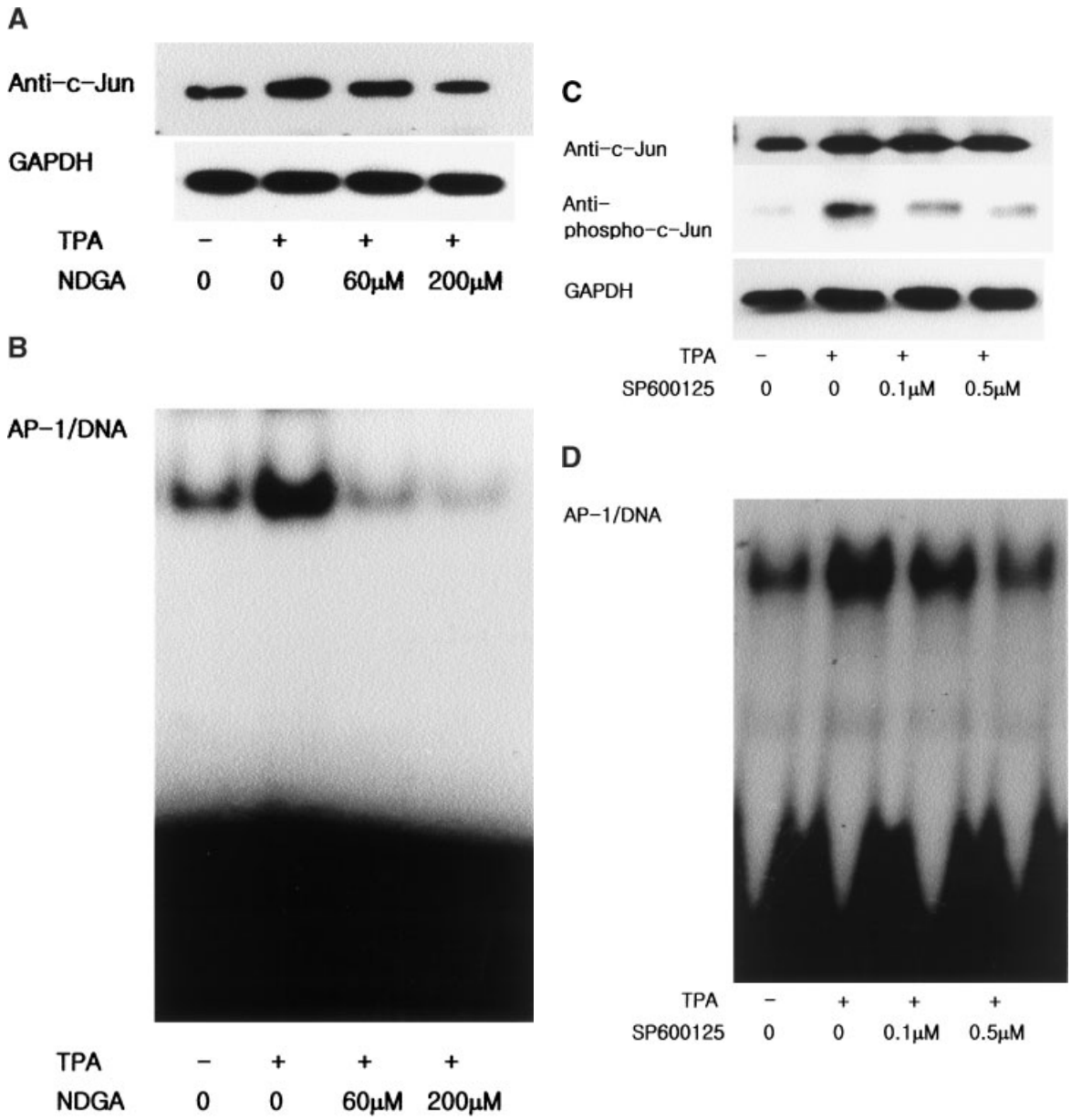


**Fig. 3.** **A**: Inhibition of TPA-induced c-jun protein level after the treatment of NDGA for 6 h. The protein levels were normalized against GAPDH levels. **B**: Inhibition of TPA-induced AP-1 binding activity after the treatment of NDGA for 6 h. **C**: Inhibition of c-jun protein phosphorylation by the treatment of SP600125 for 6 h. Results were normalized against GAPDH levels. **D**: Inhibition of binding activity between AP-1 protein and DNA by SP600125.

cleaved at the sequence Asp-Glu-Val-Asp (DEVD) into fragments of approximately 86 and 23 kDa during apoptosis by caspase 3 [Nagata, 1997; Slee et al., 1999]. Therefore, we investigated whether caspase 9 and caspase 3 were involved in apoptosis induced by AP-1 inhibitors such as NDGA and SP600125 in HL60 cells. As shown in Western blotting (Fig. 7E,F), induction of apoptosis by NDGA or SP600125 in HL60 cells was associated with activation of caspase 9 and caspase 3 and specific cleavage of its substrate PARP. After treatment of 70 μM NDGA, the proteolytic form of PARP, p85, was shown to appear at 12 h.

### c-Myc Antisense Treatment and Reversal of Apoptosis

In subsequent studies, we sought to examine the functional relevance of increased c-myc expression by AP-1 inhibitors. Previous studies

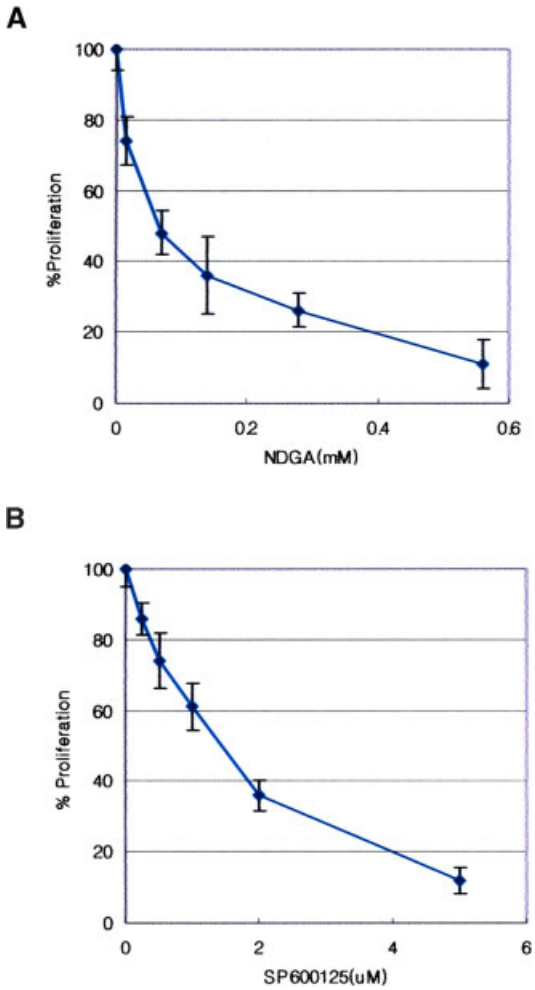


**Fig. 4.** Inhibition of HL60 cell proliferation by NDGA (**A**) and SP600125 (**B**). The percentage control growth was estimated from three independent experiments. HL60 cells were treated with the indicated concentration of NDGA or SP600125 for 24 h. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

have established that c-myc antisense oligonucleotide blocks the expression of myc [Capeans et al., 1998]. Experiments were performed to determine the effects of c-myc antisense oligonucleotide on the generation of apoptosis by AP-1 inhibitors. Prior to examining the effects of this antisense oligonucleotide on apoptosis, its ability to block endogenous c-myc expression was investigated. The endogenous c-myc RNA level was decreased in transfectants, as shown by RT-PCR (Fig. 8A). Cells were treated with NDGA or SP600125 in the presence of c-myc antisense oligonucleotide, and the AP-1 binding activity was determined by EMSA. The AP-1 binding activity was also lowered as in case of treatment by only inhibitors (NDGA or SP600125) in an EMSA experiment (Fig. 8B). However, the levels of c-myc, p53, and bax proteins were not significantly increased in Western blot analysis unlike the case of treatment by only inhibitor (Fig. 8C). When the cells were treated with NDGA or SP600125 in the presence of c-myc antisense oligonucleotide, the apoptosis symptoms were rarely observed in FACS analysis (Fig. 9). These findings demonstrated that the engagement of c-myc in an AP-1 inhibitor-mediated cellular pathway into apoptosis occurs upstream of p53 and bax, but downstream of c-jun. In conclusion, AP-1 inhibition by NDGA or SP600125 modulated the expression of p53 and bax proteins while stimulating an increase in myc protein.

## DISCUSSION

The *c-jun* gene is positively auto-regulated by its product [Angel and Karin, 1991]. The positive auto-regulation of the *c-jun* gene can function as a genetic step responsible for the conversion of early events in signal transduction into long-lasting effects on cellular gene expression [Vermes et al., 1995]. When HL60 cells were treated with TPA plus NDGA, the TPA-induction of AP-1-binding activity was clearly prevented at 30 min by NDGA (Fig. 1A). This immediate induction by TPA was cycloheximide-resistant, which is a protein synthesis inhibitor. The AP-1 binding activity was suppressed by the treatment of NDGA regardless of the presence of cycloheximide. The important observation that this inhibition against AP-1 binding occurs regardless of the presence of cycloheximide has led to the understanding that NDGA inhibits the TPA-induced post-translational promotion of c-Jun/AP-1 binding activity. TPA activates PKC, and subsequently induces AP-1 responsive gene expression. The activation of PKC results in rapid, site-specific dephosphorylation of c-Jun at sites near the carboxy terminal located just upstream of the basic region, and this is coincident with the increased AP-1 binding activity [Ofir et al., 1990]. In addition to this, the phosphorylations of two serine residues (Ser63, 73) of c-jun and a threonine residue (Thr232) of c-fos positively regulate the transactivating activity of c-jun and c-fos [Deng and Karin, 1994]. These phosphorylations are regulated by the JNK and c-Fos regulating kinase (FRK). That is, dephosphorylation of the PKC and phosphorylation of

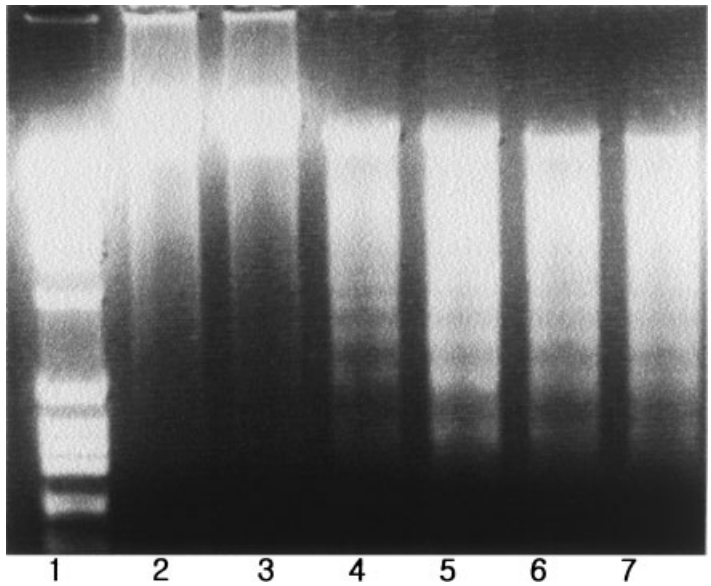


**Fig. 5.** DNA fragmentation induced by NDGA or SP600125 in HL60 cells. **Lane 1**, 100 bp marker; **lane 2**, cells treated with 70 μM NDGA for 0 h; **lane 3**, cells treated with 1.25 μM SP600125 for 0 h; **lane 4**, cells treated with 70 μM NDGA for 12 h; **lane 5**, cells treated with 70 μM NDGA for 24 h; **lane 6**, cells treated with 1.25 μM SP600125 for 12 h; **lane 7**, cells treated with 1.25 μM SP600125 for 24 h.

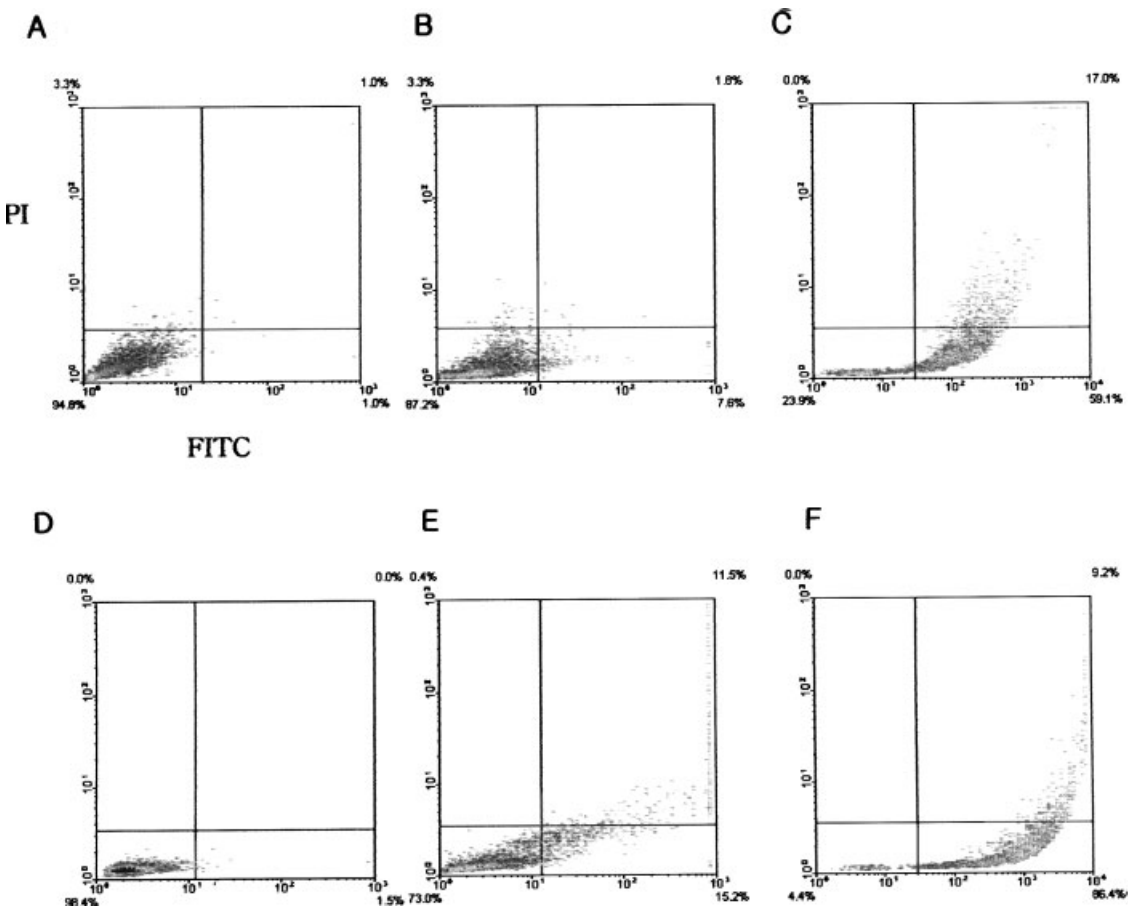


**Fig. 6.** Dot-plot of FITC-annexin V (x-axis) and propidium iodide (y-axis) flow cytometry of cells after the treatment of inhibitor. The percentages in each quadrant represent the cells versus the total. These representative data gave similar repetitive results. **A**: Control cells (untreated for 24 h). **B**: Cells treated with 20 μM NDGA for 24 h. **C**: Cells treated with 70 μM NDGA for 24 h. **D**: Control cells (untreated for 24 h). **E**: Cells treated with 0.25 μM SP600125 for 24 h. **F**: Cells treated with 1.25 μM SP600125 for 24 h.

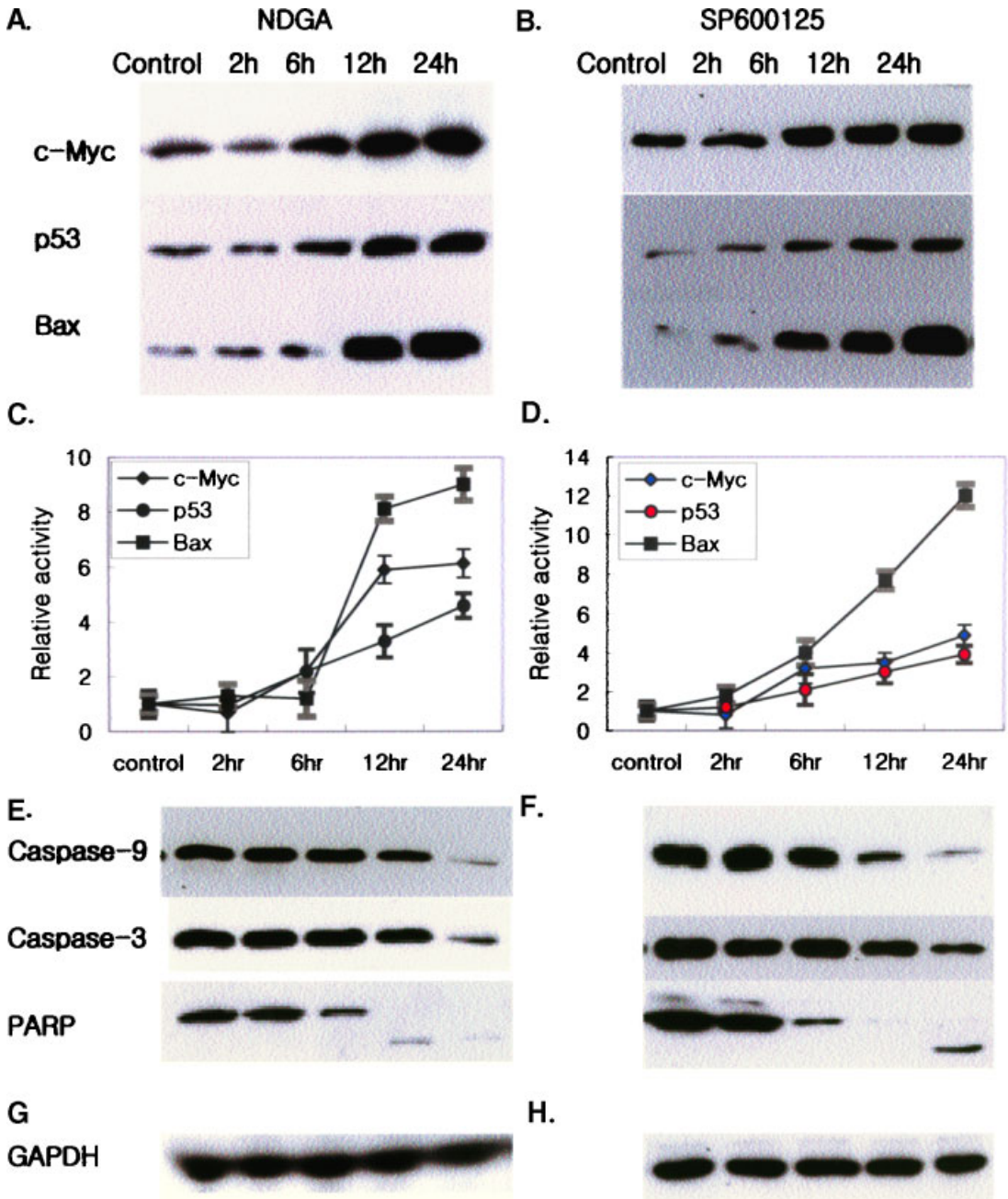


**Fig. 7.** Western blot analysis of c-myc, p53, bax, caspase 9, caspase 3, and poly[ADP-ribose]polymerase (PARP) in HL60 cells treated with 70 μM NDGA or 1.25 μM SP600125 for 2, 6, 12, and 24 h, respectively. $^{c\text{-}myc}P < 0.005$ (two way ANOVA), $^{p53}P < 0.075$, $^{bax}P < 0.05$. The statistically significant difference between before and after 12 h of inhibitor treatment was observed. All the results were normalized against GAPDH levels (**G**, **H**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

JNK and FRK may enhance the protein's ability to stimulate DNA-binding activity and gene transcription. The experiment using the in vitro expressed and purified c-fos and c-jun protein (Fig. 1B) shows that NDGA blocked jun/fos-DNA binding indicating an $IC_{50}$ of 27 μM under the concentration of each 0.5 μM Jun and fos protein. Each B-Zip domain of jun and fos protein used in Figure 1B has only a basic region and a leucine zipper region, not including dephosphorylation and phosphorylation sites activated by PKC and JNK or FRK, respectively. Thus, the possible inhibition sites of NDGA are limited to both basic regions and the

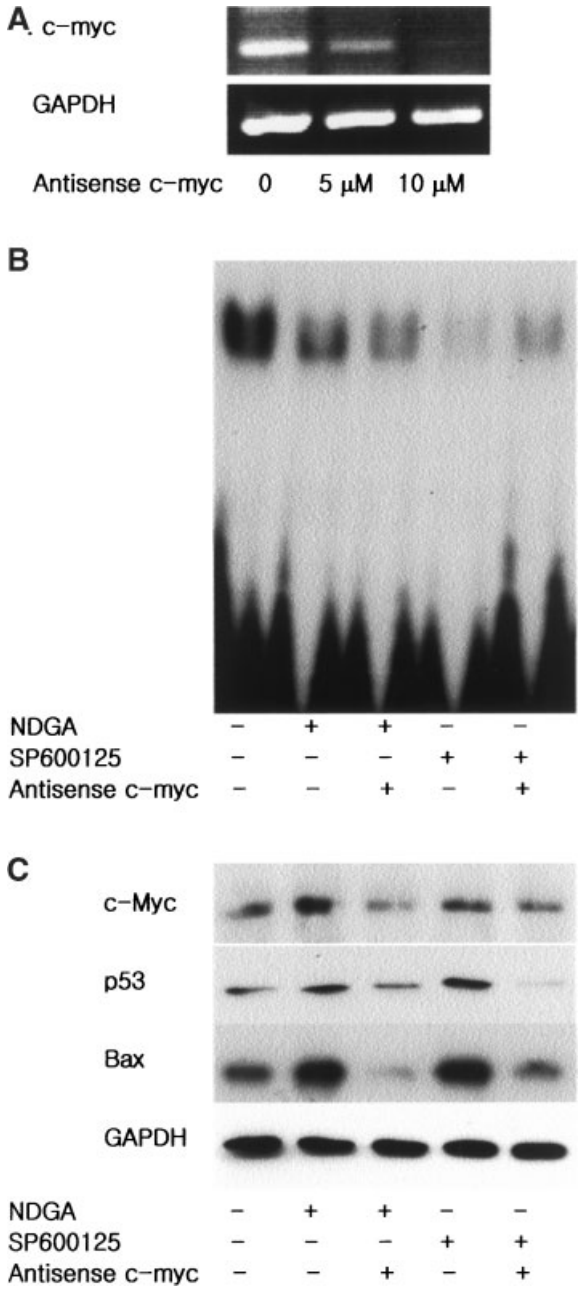


**Fig. 8.** **A**: RT-PCR of *myc* genes from HL60 cells. **B**: Analysis of jun-fos dimer binding with the AP-1 oligonucleotide in EMSA. After the treatment of 70 μM NDGA or 1.25 μM SP600125 with or without 10 μM antisense c-myc to the confluent cells, equal amounts of HL60 cell extracts were applied to the EMSA. The antisense c-myc oligomer did not affect the reduction of AP-1 activity by NDGA or SP600125. **C**: Western blot analysis of c-myc, p53, and bax in HL60 cells treated with 70 μM NDGA or 1.25 μM SP600125, with or without 10 μM antisense c-myc for the indicated time. Results were normalized against GAPDH levels.

leucine zippers of c-jun and c-fos proteins. The inhibitory action of NDGA on jun/AP-1 and DNA binding in cell nuclear extracts may not be via blocking of the regulatory domain at c-jun sites that positively regulate its DNA-binding activity, but via direct interference of dimer binding with DNA.

NDGA also inhibited a de novo synthesis of c-jun/AP-1, as shown in Figure 2. The positive auto-regulation mechanisms of c-jun are well explained in this mRNA expression study. NDGA, which has an inhibitory mechanism blocking the interaction between the AP-1 protein and the AP-1 site of DNA, also showed inhibitory action on the c-jun mRNA expression.

There is indication that NDGA shows selectivity on AP-1/DNA binding versus the other transcription factor, myc-max/DNA binding. $IC_{50}$ values for this compound on AP-1/DNA binding and myc-max/DNA binding are determined to be 27 μM versus 8 mM, respectively. These studies were performed on the recombinant proteins.

Recent studies have shown that JNK inhibitor SP600125 (anthrax[1,9-cd]pyrazol-6(2H)-one) completely blocked IL-induced accumulation of phospho-jun and induction of c-jun transcription [Han et al., 2001]. In order to explain that NDGA induces apoptosis because of effects on AP-1 other than proteins, we used SP600125, which resulted in inhibition of AP-1 activity. In our experiments, SP600125 dose dependently inhibited the phosphorylation of c-jun, and prevented AP-1 binding activity in TPA-induced HL60 cells. SP600125 also reveals that the dose response for inhibition of AP-1 activity corresponds to that for induction of apoptosis effects on HL60 cells. The strongest data implicating induction of c-myc, p53, and bax in the promotion of apoptosis by AP-1 inhibitor, appears in Figure 7, supported by both NDGA and SP600125.

Previous studies have provided evidence that c-myc is required for efficient response to a variety of apoptotic stimuli, including transcription and translation inhibitors, glucose deprival, heat shock, chemotoxins, DNA damage, and cancer chemotherapeutics [Prendergast, 1999]. It has been also proposed that c-myc acts to sensitize cells to a variety of apoptotic triggers [Capeans et al., 1998]. Our studies of HL60 cancer cells demonstrate that the inhibition of AP-1 activity by NDGA or SP600125 induces apoptosis. This apoptosis is

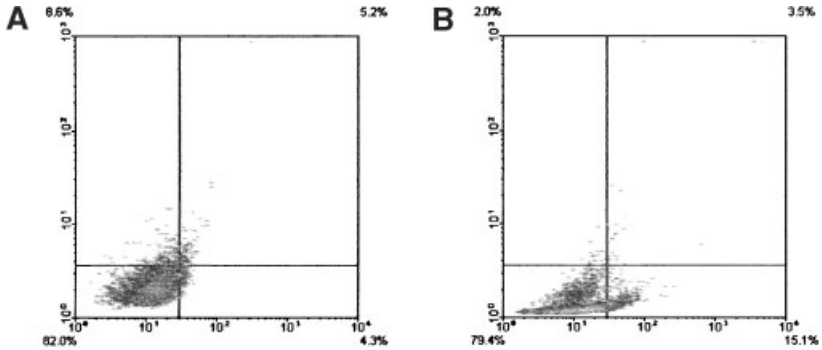


**Fig. 9.** Dot-plot of FITC-annexin V (x-axis) and propidium iodide (y-axis) flow cytometry of cells showing the blocking of NDGA-related apoptosis after the treatment of NDGA with antisense c-myc. The percentage in each quadrant represents the cells versus the total. These representative data gave similar repetitive results. **A**: Cells treated with 70 μM NDGA and 10 μM antisense c-myc for 24 h. **B**: Cells treated with both 1.25 μM SP600125 and 10 μM antisense c-myc for 24 h.

accompanied by activation of bax, caspase 9, and caspase 3 proteins, with alteration of myc and p53 levels, suggesting that the apoptosis may occur via a myc-dependent pathway. Our results also support such an observation, by demonstrating that the down-regulation of myc expression using antisense oligomer restored the apoptosis induced by AP-1 inhibition and restored the p53 and bax expression. When the antisense c-myc oligonucleotide was applied to the cells, the protein levels of p53 and bax were also lowered, demonstrating that p53 and bax might be downstream of myc and AP-1. The finding that myc antisense reverses apoptosis (Fig. 9) with concomitant suppression of p53 and bax (Fig. 8) strongly implicates myc in apoptosis induction by AP-1 inhibitor. Although these results should be further investigated using p53-mutated cells to be relevant to other cell models, AP-1 inhibitor NDGA not only inhibited AP-1 binding but also induced apoptosis in another human cell line, A549 cells from our results. A suggested model for the c-myc function proposed that c-myc activates proliferation and primes apoptosis through one pathway and triggers apoptosis through a mechanically separated second pathway [Prendergast, 1999]. We here suggest a model for the presence of the AP-1 signal as a survival factor that determines the outcome of myc-induced proliferation or apoptosis, as shown in Figure 10.

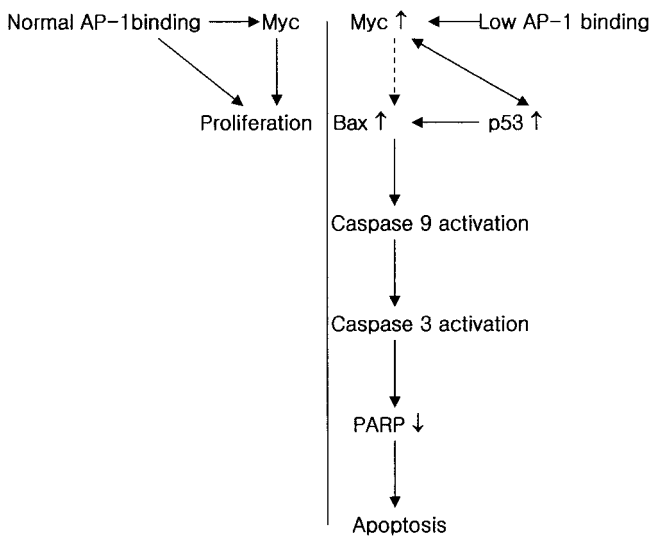


**Fig. 10.** The possible pathways involved in myc-related apoptosis induced by AP-1 inhibitor in HL60 cells.

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