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Role of Protein Tyrosine Phosphorylation in Etoposide-Induced Apoptosis and NF-κB Activation

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ABSTRACT. When a human myeloid cell line, U937, was incubated with etoposide (10 μg/mL), morphologically apoptotic cells first appeared at 3 hr and increased with time to 50% at 6 hr. Pretreatment of U937 cells for 30 min with a potent tyrosine kinase inhibitor, herbimycin A (10 μM), significantly suppressed the appearance of apoptotic morphological changes. Concomitantly, herbimycin A pretreatment prevented both high molecular weight and internucleosomal DNA fragmentation induced by etoposide. Two major bands at 30 and 66 kDa with enhanced tyrosine phosphorylation inhibited by herbimycin A were detectable after 30 min of incubation with etoposide. In addition, herbimycin A prevented etoposide-induced NF-κB activation. The expressions of Bcl-2 and Bax, on the other hand, were not affected by herbimycin A pretreatment. Herbimycin A was also found to inhibit 1-β-D-arabinofuranosylcytosine-induced apoptotic changes and NF-κB activation. These results suggest that activation of tyrosine kinase(s) may play an important role in apoptotic processes induced by a variety of anti-cancer drugs. BIOCHEM PHARMACOL **55**;2:185–191, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. apoptosis; tyrosine phosphorylation; herbimycin A; etoposide; ara-C; NF-κB

Apoptosis is a common form of cell death induced by a wide variety of anti-cancer drugs [1]. Apoptosis is known to culminate in a typical morphological change characterized by nuclear condensation and internucleosomal DNA fragmentation [2], but the intracellular signaling pathways involved in the process have not been clarified. Previous investigations have suggested the pivotal role of various protein kinases, including protein kinase C [3], protein kinase A [4], or tyrosine kinase [5]. The role played by tyrosine kinase(s) in the apoptotic process has been evaluated with the use of a specific inhibitor, herbimycin A [6]. For example, Lee *et al.* [7] demonstrated that dexamethasone-induced apoptosis is inhibited by herbimycin A in rat thymocytes. Another inhibitor of tyrosine kinase, genistein, has been reported to prevent taxol-induced apoptosis in U937 cells [8]. However, the precise mechanism(s) whereby herbimycin A inhibits apoptosis induced by anti-cancer drugs has not been defined clearly.

Several intracellular events have been reported to precede the appearance of morphological apoptosis. Among them is an activation of a transcriptional factor, NF-κB, which participates in the induction of numerous cellular and viral genes [9]. A variety of agents, including anti-cancer drugs [10–12], glucocorticoid [13], TNF-α† [14], and anti-Fas [15], that are able to induce apoptosis have been reported to activate NF-κB. The exact link of NF-κB activation with the occurrence of apoptosis, however, has not been identified. In the cytoplasm, an inhibitory subunit, IκBα, binds to NF-κB complexes and makes them inactive [9]. Upon stimulation, IκBα is cleaved mainly through its phosphorylation and is dissociated from the complex. Although several *in vitro* studies have suggested that protein kinase C is one of the kinases responsible for the phosphorylation of IκBα [16, 17], a kinase(s) to phosphorylate IκBα in *in vivo* situations remains to be identified. Notably, recent works have suggested that hypoxia [18] or lipopolysaccharide and interferon γ [19] cause the activation of NF-κB through phosphorylation on tyrosine residues.

On the basis of the notion described above, in the present study we investigated the effects of herbimycin A on: (i) etoposide-induced apoptosis, as judged by several parameters, including morphology, HMW, and internucleosomal DNA fragmentation, and (ii) protein tyrosine phosphorylation and NF-κB activation following treatment with etoposide, to better understand the role of tyrosine kinase activation during apoptosis.

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† *Abbreviations:* CHEF, clamped homogeneous electric field; ara-C, 1-β-D-arabino-furanosylcytosine; HMW, high molecular weight; PDTC, pyrrolidine dithiocarbamate; and TNF-α, tumor necrosis factor-α.

MATERIALS AND METHODS

Cell Culture and Determination of Morphologically Apoptotic Cells Following Drug Exposure

A human myeloid leukemia cell line, U937, was provided by the Japanese Cancer Resources Bank. U937 was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL). The culture was maintained at 37° in a humidified atmosphere of 5% CO_2/95% air. For the morphological assay for apoptosis, cells at a density of 2×10^5/mL in their logarithmic growth phase were preincubated for 30 min with or without herbimycin A. Etoposide was then added to the culture without washings. After various lengths of incubation, 100 μL of the cells was placed in a cytospin centrifuge and centrifuged on a glass slide at $40 \times g$ for 2 min. Apoptotic cells in May–Grünwald–Giemsa-stained preparations were evaluated by scoring those cells that contained fragmented nuclei and condensed chromatin [20].

Preparation of DNA for Gel Electrophoresis

Cell pellets (3×10^6 cells) with or without drug treatment were resuspended in 100 μL of a solution containing 10 mM Tris, 20 mM NaCl, 50 mM EDTA, plus 100 μL of prewarmed 2% low-melting-point agarose. Plugs were polymerized at 4° for 10 min, transferred to a solution containing 100 mM EDTA, pH 8.0, 1% sodium lauryl sarcosine, and 1 mg/mL proteinase K, followed by overnight incubation at 50° without agitation. Plugs and supernatants were then separated. The DNA in the supernatants was prepared and used for conventional agarose gel electrophoresis by the method described below [21]. The plugs were washed four times in a washing buffer containing 20 mM Tris and 50 mM EDTA and stored at 4° in the washing buffer until they were examined by pulsed-field gel electrophoresis.

Assay for HMW and Internucleosomal DNA Fragmentation

To resolve HMW DNA fragments, we used CHEF gel electrophoresis. CHEF electrophoresis was carried out with a CHEF-DR II purchased from Bio-Rad Laboratories. Horizontal gels of 1.5% agarose were run at 6 V/cm with a ramped switch time of 0.5 to 10 sec for 9.5 hr followed by a ramped switch time of 10–60 sec for 9.5 hr in 0.25 × TAE (10 mM Tris, 10 mM acetate, 0.5 mM EDTA) at 14°. Two sets of pulse markers were used as standards: (i) 0.5 to 23 kbp fragments consisting of λDNA *Hin*dIII fragments; (ii) 225–2200 kbp consisting of chromosomes isolated from *Saccharomyces cerevisiae*. DNA gels were stained with ethidium bromide and photographed in UV light with a Polaroid camera [21]. For the measurement of internucleosomal DNA fragmentation, approximately 10 μg DNA was placed on a 1.8% agarose gel containing 0.5 μg/mL ethidium bromide. Electrophoresis was carried out at 3 V/cm for 3 hr, and photographs were taken under UV light [20].

Western Blotting

After incubation with the drug(s) for various intervals, the cells (4×10^6) were collected by centrifugation, washed in ice-cold PBS, and lysed in 400 μL of a lysis buffer [10 mM Tris–HCl (pH 7.4), 5 mM EDTA, 0.15 N NaCl, 0.1% SDS, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM orthovanadate, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL leupeptin] at 4° with sonication. Protein concentration was measured by the Bio-Rad protein assay kit (Bio-Rad). SDS-sample buffer [65 mM Tris–HCl (pH 6.7), 3.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.2% bromophenol blue] was then added to each 40 μg of postlysate protein, which was boiled for 3 min and electrophoresed onto a 12% SDS-polyacrylamide gel. The proteins were electrophoretically transferred from the gel onto an Immobilon-P, polyvinylidene difluoride membrane (Millipore) [22]. After blocking with 1% bovine serum albumin in TBST (10 mM Tris, 150 mM NaCl, 0.07% Tween 20), they were immunoblotted using mouse anti-phosphotyrosine monoclonal antibody (PY20), or mouse anti-Bcl-2 monoclonal antibody (DAKO A/S), or goat anti-Bax polyclonal antibody at a 1:500 dilution [23]. The primary antibody was detected using goat anti-mouse horseradish peroxidase-conjugated IgG or donkey anti-goat horseradish peroxidase-conjugated IgG (CHEMICON) at a 1:500 dilution and the ECL chemiluminescent detection system (Amersham).

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared according to the methods described by Li *et al.* [24] with slight modifications. A 26-bp synthetic oligonucleotide (5′-GATCCAGAGGGGACTT TCCGAGAGGC-3′) containing an NF-κB binding site (GGGGACTTTCC) was labeled with [α-^{32}P]dGTP and Klenow enzyme [25]. The labeled DNA was purified through a NICK Column (Pharmacia). Binding reactions with equal amounts of nuclear extracts (4 μg/reaction) were performed in a 10-μL final volume of 12% glycerol, 12 mM HEPES–NaOH, 4 mM Tris–HCl, 60 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, pH 7.9, containing a 10,000 cpm probe, 2 μg poly dIdC, and 3 μg bovine serum albumin. After incubation for 30 min at room temperature, the mixtures were loaded on a 4% polyacrylamide gel in TAE buffer (1 × TAE: 6.7 mM Tris, 3.3 mM sodium acetate, 1 mM EDTA, pH 7.9). After electrophoresis, the dried gel was autoradiographed. For competition assay, unlabeled NF-κB oligo-

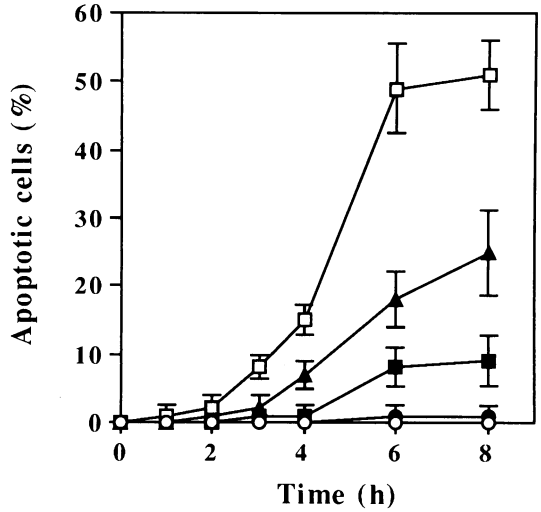

FIG. 1. Effect of herbimycin A on etoposide-induced apoptosis in U937 cells. U937 cells were preincubated with either medium alone or herbimycin A for 30 min, followed by further incubation with etoposide (10 μg/mL). Cells were taken at the indicated time for the measurement of apoptotic cells morphologically. Data represent the means ± SD of three separate experiments. Key: (○) no treatment; (□) etoposide only; (▲) etoposide plus 1 μM herbimycin A; (■) etoposide plus 5 μM herbimycin A; and (●) etoposide plus 10 μM herbimycin A.

nucleotide was added in an amount 100 times that of the end-labeled fragment [11].

RESULTS

Inhibition of Etoposide-Induced Morphological Apoptosis and DNA Fragmentation by Herbimycin A

We first examined the time–course of the appearance of apoptotic cells following incubation with etoposide (10 μg/mL) and/or herbimycin A (1, 5, or 10 μM). As indicated in Fig. 1, etoposide alone could induce significant numbers of apoptotic cells after 3 hr of incubation; the proportion of apoptotic cells reached 50% after 6 hr. Pretreatment with herbimycin A for 30 min significantly prevented apoptosis in a concentration-dependent manner (Fig. 1). Notably, even if the incubation was prolonged up to 8 hr, we found no cells exhibiting the morphological changes of apoptosis with 10 μM herbimycin A. Two kinds of DNA fragmentation, HMW [26] and internucleosomal [2], have been reported during the apoptotic process. In fact, etoposide induced distinct HMW and internucleosomal DNA fragmentation after 3 hr of incubation (Fig. 2). Figure 2 also demonstrates that herbimycin A pretreatment completely abolished both types of DNA fragmentation during at least 3 hr of incubation. A kinetic study for the time of addition of herbimycin A revealed that this drug was completely effective in preventing apoptosis, even if added 30 min after the start of incubation with etoposide (Fig. 3). This fact indicates that herbimycin A does not interfere with the initial interaction between etoposide and DNA.

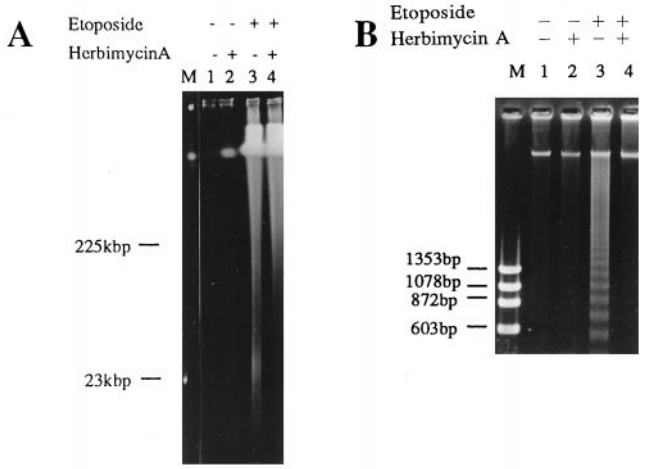

FIG. 2. Effect of herbimycin A on etoposide-induced HMW and internucleosomal DNA fragmentation. U937 cells were preincubated with either medium alone or herbimycin A (10 μM) for 30 min. After further incubation with etoposide (10 μg/mL) for 3 hr, DNA was extracted and subjected to HMW (A) and internucleosomal DNA fragmentation assay (B). Lane M: molecular weight size marker; lane 1: no treatment; lane 2: herbimycin A; lane 3: etoposide; and lane 4: etoposide plus herbimycin A.

Protein Tyrosine Phosphorylation after Incubation with Etoposide

Exposure of U937 cells to etoposide for various intervals stimulated tyrosine phosphorylation of two phosphoproteins (Fig. 4). These have approximate molecular masses of 30 and 66 kDa. A kinetic study revealed that tyrosine phosphorylation of these proteins was first apparent after 30

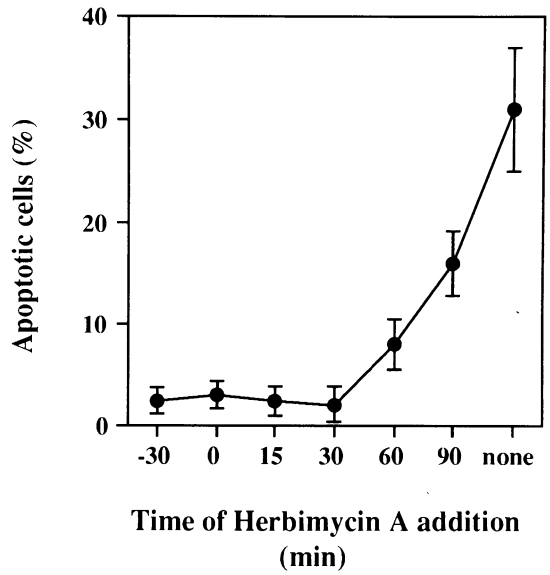

FIG. 3. Effect of the time of herbimycin A addition on apoptosis induced by etoposide. Etoposide (10 μg/mL) was added to U937 cells at time zero. Herbimycin A (10 μM) was added to the culture at the indicated times; "−30" indicates that the cells were preincubated with herbimycin A for 30 min before the addition of etoposide. Data represent the means ± SD of three separate experiments.

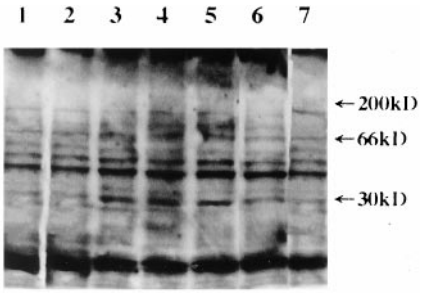

FIG. 4. Kinetics of etoposide-induced protein tyrosine phosphorylation. U937 cells were incubated with 10 μg/mL etoposide for various intervals. Cells were lysed, resolved by SDS–PAGE, and immunoblotted with anti-phosphotyrosine monoclonal antibody. Lane 1: no treatment; lane 2: 3 min; lane 3: 30 min; lane 4: 60 min; lane 5: 120 min; lane 6: 60 min after preincubation of herbimycin A (10 μM) for 30 min; and lane 7: herbimycin A only for 90 min.

min, reaching its peak at 60 min. Such enhanced tyrosine phosphorylation was abolished by the pretreatment of cells with herbimycin A for 30 min.

Bcl-2 and Bax Expression

The ratio of Bax to Bcl-2 is considered to play a critical role in regulating cell survival when cells are stimulated by a variety of apoptosis inducers [27]. The expression of Bcl-2 and Bax was quite stable at different intervals following incubation with etoposide. Moreover, pretreatment with herbimycin A did not have any effects on the expression of these proteins in cells incubated with etoposide (Fig. 5).

Inhibition of Etoposide-Induced NF-κB Activation by Herbimycin A

We next tried to determine whether herbimycin A can inhibit NF-κB activation mediated by etoposide. NF-κB activation in U937 nuclear cell extracts was first detected at 1 hr following exposure to etoposide, reached its peak at

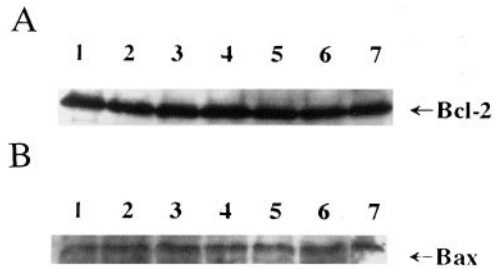

FIG. 5. Bcl-2 (A) and Bax (B) expression during etoposide and/or herbimycin A treatment. U937 cells were preincubated with either medium alone or herbimycin A (10 μM) for 30 min. After further incubation with etoposide (10 μg/mL) for various intervals, cell extracts were prepared. Western blotting was carried out by using a specific antibody against Bcl-2 or Bax protein. Lane 1: no treatment; lane 2: 3 min; lane 3: 30 min; lane 4: 60 min; lane 5: herbimycin A only for 60 min; lane 6: 60 min after preincubation of herbimycin A for 30 min; and lane 7: Raji cells.

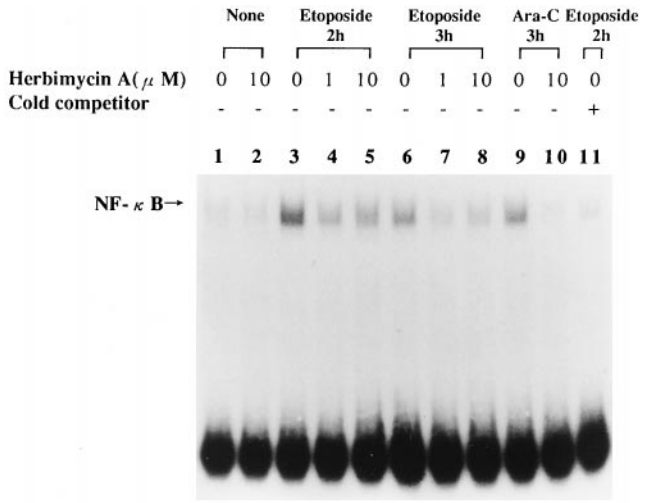

FIG. 6. Effect of herbimycin A on NF-κB activation induced by etoposide or ara-C. U937 cells were preincubated with either medium alone or herbimycin A (10 μM) for 30 min. After further incubation with etoposide (10 μg/mL) or ara-C (10 μM) for 2 or 3 hr, nuclear extracts were prepared and used for electrophoretic mobility shift assay.

2 hr, and was subsequently down-regulated (data not shown). This process was blocked completely by the addition of cold competitors. Furthermore, the supershifts of the bands for NF-κB after treatment with anti-NF-κB p50 or p65 antibody confirmed the identity of NF-κB complexes (data not shown). Herbimycin A (10 μM) prevented NF-κB activation almost completely, at least during 3 hr of incubation with etoposide (Fig. 6).

Effect of Herbimycin A on ara-C-Induced Apoptosis and NF-κB Activation

We attempted to examine whether or not the inhibitory effect of herbimycin A on apoptosis and NF-κB activation is specific for etoposide by using another well-known apoptosis-inducer, ara-C. As can be seen from Fig. 7, herbimycin A was found to inhibit morphological apoptosis, and HMW and internucleosomal DNA fragmentation. Concomitantly, ara-C-induced NF-κB activation was suppressed (Fig. 6).

Effect of Genistein on Etoposide- or ara-C-Induced Apoptosis and NF-κB Activation

Genistein is another potent and specific inhibitor of tyrosine kinase [28]. Genistein (20 μg/mL) pretreatment for 30 min prevented morphological apoptosis induced by etoposide (10 μg/mL) or ara-C (10 μM) for up to 8 hr of incubation (Fig. 8A). When examined after 4 hr of incubation with these apoptosis inducers, genistein inhibited completely both HMW and internucleosomal DNA fragmentation (Fig. 8, B and C). Finally, genistein was found to abolish etoposide-induced NF-κB activation (Fig. 8D).

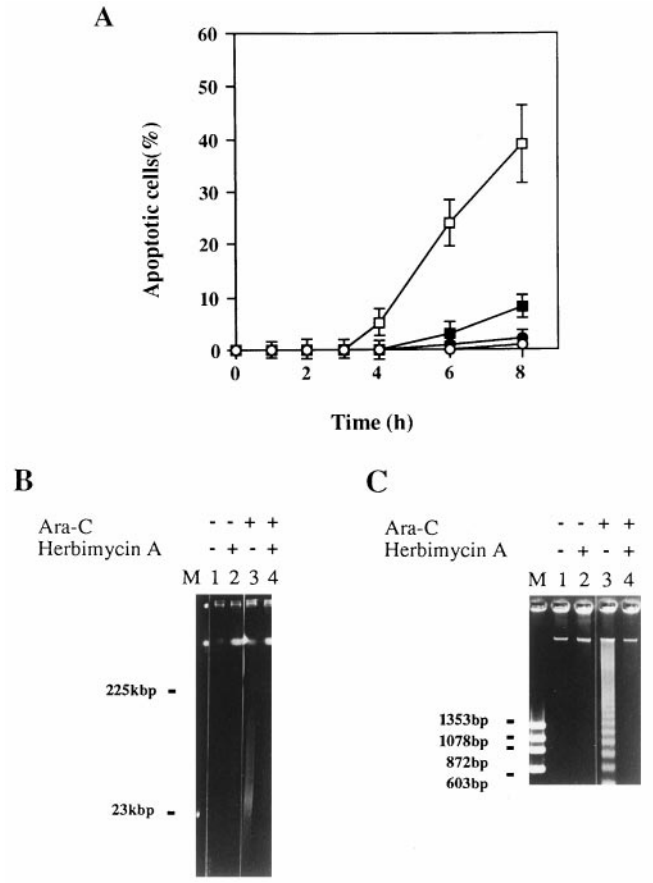

FIG. 7. Effect of herbimycin A on ara-C-induced apoptosis and DNA fragmentation. After preincubation with either medium alone or herbimycin A (5 or 10 μM) for 30 min, ara-C was added to the culture at a concentration of 10 μM. Cells were then taken at the indicated times for apoptotic cell analysis (A). Data represent the means ± SD of three separate experiments. Key: (○) no treatment; (□) ara-C only; (■) ara-C plus 5 μM herbimycin A; and (●) ara-C plus 10 μM herbimycin A. A portion of the cells was harvested after 4 hr of incubation and used for HMW (B) and internucleosomal (C) DNA fragmentation assay. Lane M: molecular weight size marker; lane 1: no treatment; lane 2: herbimycin A (10 μM); lane 3: ara-C; and lane 4: ara-C plus herbimycin A.

DISCUSSION

In the present study, we have shown clearly that a potent tyrosine kinase inhibitor, herbimycin A, inhibits apoptosis induced by etoposide or ara-C. We have also found that genistein, another inhibitor of tyrosine kinase, has the same effect. These results are in accordance with previous reports, demonstrating the inhibitory effects of herbimycin A on apoptosis induced by dexamethasone [7] or TNF-α [29]. Recently, Ponnathpur *et al.* [8] indicated that genistein reduces taxol-induced apoptosis in human leukemic cells. Our data, however, show that herbimycin A delays the onset of apoptosis rather than completely blocks it. Since the previous reports noted above used only one time point for the evaluation of apoptosis, it is difficult to compare our findings with those of others. Yin and Schimke [23] have shown a similar delay, but not a complete prevention, of

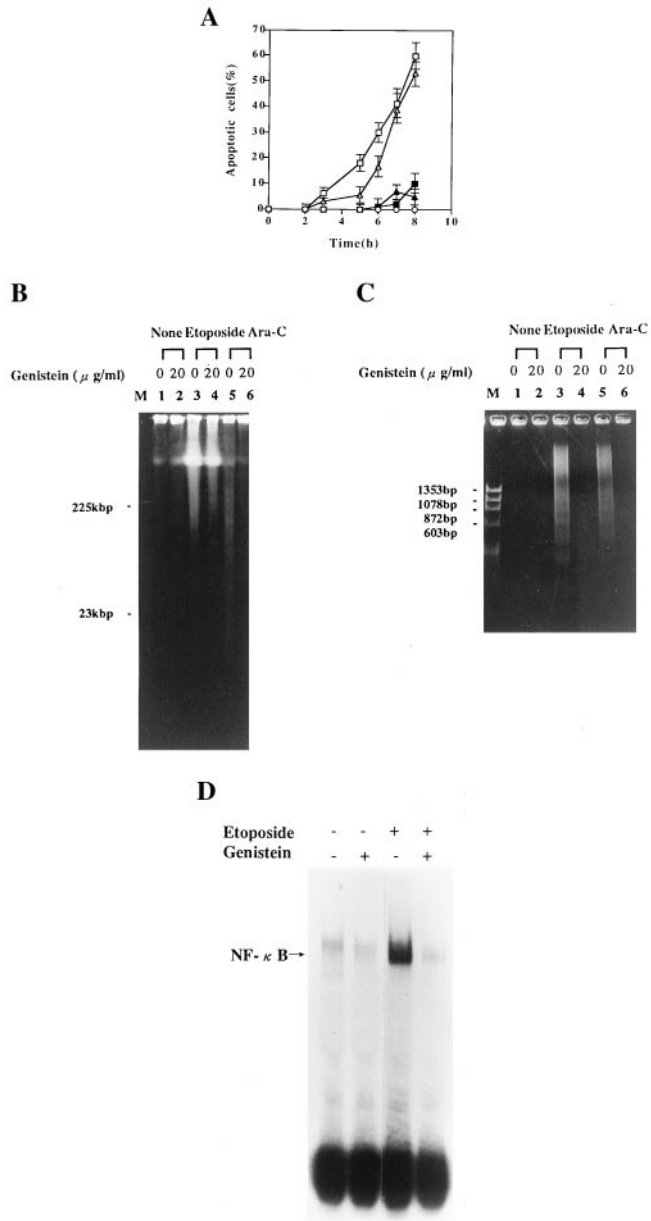

FIG. 8. Effect of genistein on etoposide- or ara-C-induced morphological apoptosis, DNA fragmentation, and NF-κB activation. After preincubation with either medium alone or genistein (20 μg/mL) for 30 min, etoposide or ara-C was added to the culture at a concentration of 10 μg/mL or 10 μM, respectively. Cells were then taken at the indicated times for apoptotic cell analysis (A). Data represent the means ± SD of three separate experiments. Key: (○) no treatment; (□) etoposide only; (■) etoposide plus genistein; (△) ara-C only; and (▲) ara-C plus genistein. A portion of the cells was harvested after 4 hr of incubation and used for HMW (B) and internucleosomal (C) DNA fragmentation assay. Lane M: molecular weight size marker; lane 1: no treatment; lane 2: genistein; lane 3: etoposide; lane 4: etoposide plus genistein; lane 5: ara-C; and lane 6: ara-C plus genistein. (D) U937 cells were preincubated with either medium alone or genistein (20 μg/mL) for 30 min. After further incubation with etoposide (10 μg/mL) for 2 hr, nuclear extracts were prepared and used for electrophoretic mobility shift assay.

apoptosis in *bcl-2* transfected cells. As possible explanations, we speculate that (i) there may be another apoptosis pathway that bypasses the activation of tyrosine kinase, or (ii) residual tyrosine kinase activity activates the apoptosis pathway because of the insufficient inhibition of kinase in our system.

The tyrosine phosphorylation of various proteins during apoptosis has been described. Migita and coworkers [30] have demonstrated the tyrosine phosphorylation of pp100, pp94, pp74, and pp21 proteins following the stimulation of thymocytes by anti-CD3 monoclonal antibody cross-linking. Increased tyrosine phosphorylation at about 56–60, 70, and 100–110 kDa was detected in a human T-cell line, Jurkat, during Fas-mediated apoptosis [31]. The authors have presented the possibility that the 110 kDa tyrosine-phosphorylated protein is identical with c-cbl. Uckun *et al.* [5] have reported that ionizing radiation distinctly enhanced the tyrosine phosphorylation of multiple proteins with molecular masses of 34, 55, 69, 76, 97, and 150 kDa in human B-lymphocyte precursors. In the current study, we have demonstrated tyrosine phosphorylation on phosphoproteins of approximately 30 and 66 kDa. The nature of these proteins is as yet unidentified. Most significantly, our findings regarding the kinetics of the appearance of tyrosine phosphorylation differ from those of previous studies. Namely, other investigators have noted increased tyrosine phosphorylation within a few minutes following the addition of apoptotic stimuli [15, 30, 31]. The tyrosine phosphorylation following etoposide treatment was, however, first detectable at 30 min, reaching its peak at 60 min. This is in accord with the time–course of apoptosis inhibition by herbimycin A in Fig. 3.

Several investigators have described NF-κB activation during apoptosis when induced by a variety of agents including ara-C [10, 12], TNF-α [14], and glucocorticoids [13]. Our group has also demonstrated that an antioxidant, PDTC, inhibits etoposide- or ara-C-induced apoptosis and concomitantly blocks NF-κB activation in HL-60 cells [11]. Furthermore, an association of high levels of c-Rel, a member of the NF-κB family, with programmed cell death in the developing avian embryo and in bone marrow cells has been reported [32]. On the other hand, recent findings have presented an anti-apoptotic role for NF-κB in apoptosis. For example, Wu *et al.* [33] have demonstrated that inhibition of NF-κB/Rel induces apoptosis in murine B cells. Other groups also reported that NF-κB activation in other cell types blocks apoptosis induced by TNF-α, ionizing radiation, or daunorubicin [34, 35]. Finally, PDTC is unable to inhibit NF-κB activation, despite a pronounced inhibition of apoptotic DNA fragmentation induced by etoposide in thymocytes [36]. The authors proposed that NF-κB activation may play a pivotal role in apoptosis in some biological systems, but it is not a general feature of the process [37].

Lin *et al.* [38] have reported that the overexpression of Bcl-2 protein inhibits both Sindbis virus-induced apoptosis and NF-κB activation in a prostate carcinoma cell line. However, Albrecht *et al.* [39] have shown that Bcl-2 overexpression prevents apoptotic cell death without interfering with the activation of NF-κB by TNF-α. We found in the present study that the expression of Bcl-2 and Bax proteins was unaffected after the addition of herbimycin A. However, considering the recent observation that the function of Bcl-2-related proteins is regulated through its phosphorylation [40], further studies are necessary to determine the effect of herbimycin A on the phosphorylation status of these proteins.

In summary, we have demonstrated an important role of protein tyrosine phosphorylation in apoptosis induced by etoposide or ara-C. Tyrosine kinases are largely classified into two groups: receptor-associated and cytoplasmic [41]. Thus, the delineation of the kinase(s) responsible for tyrosine phosphorylation in apoptosis and NF-κB activation will give us the opportunity to understand the signaling pathway of apoptosis.

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