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Prevention of Etoposide-Induced Apoptosis by Proteasome Inhibitors in a Human Leukemic Cell Line but Not in Fresh Acute Leukemia Blasts

A DIFFERENTIAL ROLE OF NF-κB ACTIVATION

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ABSTRACT. Recent research indicates that the proteasome is one of the non-caspase proteases involved in apoptotic signaling pathways. Nuclear factor-κB (NF-κB) activation, one of the key factors in apoptosis, can be prevented through abrogation of IκBα degradation by proteasome inhibition. We have investigated the effects of the proteasome inhibitors carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal (MG132) and *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (LLnL) on apoptosis and NF-κB activation induced by etoposide, using a human leukemia cell line (U937) and leukemia blasts freshly isolated from patients with acute leukemia. Pretreatment of U937 cells with MG132 or LLnL inhibited etoposide-induced morphological apoptosis and caspase-3 activation. Furthermore, MG132 or LLnL prevented NF-κB activation and IκBα degradation, but not IκBα phosphorylation at Ser32. Other inhibitors of NF-κB activation, including pyrrolidine dithiocarbamate (an antioxidant) and the peptide SN50 (an inhibitor of translocation of activated NF-κB into the nucleus), also attenuated etoposide-induced apoptosis. In leukemia blasts, although proteasome inhibitors suppressed NF-κB activation induced by etoposide, they were unable to prevent morphological apoptosis. Moreover, proteasome inhibitors by themselves caused apoptosis in leukemia blasts at the concentrations employed in this study. These results suggest that the role that NF-κB plays in apoptosis induced by etoposide in a human leukemia cell line may be different from the role it plays in freshly isolated leukemia blasts. BIOCHEM PHARMACOL **60**;6:823–830, 2000.

KEY WORDS. apoptosis; NF-κB; IκBα; proteasome; etoposide; leukemia cells

Proteolytic mechanisms play an essential role in signaling pathways involved in apoptosis. Among them, a family of cysteine proteases called caspases is indispensable for the initiation and execution of apoptosis. Activation of effector caspases through proteolysis leads to the cleavage of proteins crucial for DNA repair and maintenance of the cytoskeleton, subsequently resulting in internucleosomal DNA fragmentation and morphological changes characterized by chromatin condensation, nuclear fragmentation, and membrane blebbing [1]. Such apoptotic processes can be induced by various anticancer drugs [2, 3].

The proteasome is a large multisubunit proteolytic complex that participates in the degradation of proteins critical for cell cycle regulation, cell proliferation, and gene regulation [4]. Recent studies using proteasome inhibitors have revealed that the proteasome is one of the non-caspase proteases involved in apoptosis. Inhibition of proteasomes has been shown to induce apoptosis in Jurkat T cells [5] and human glioma cells [6], while other investigators have indicated that proteasome inhibitors attenuate some forms of apoptosis [7, 8]. From the therapeutic point of view, recent reports have suggested that proteasome inhibitors can overcome drug resistance [9–11]. This effect is thought to be mediated through prevention of activation of NF-κB† by proteasome inhibition. NF-κB participates in induction of various genes encoding cytokines, acute phase response protein, and viruses [12]. NF-κB is now thought to be one of the key factors involved in apoptotic pathways, although whether NF-κB works as a proapoptotic factor or an antiapoptotic factor depends on the cell type or inducers (reviewed in Ref. 13). NF-κB is regulated post-translationally by its inhibitory proteins, termed IκBs. Among them, IκBα, the best-characterized IκB, appears to be a primary regulator of NF-κB. Under basal conditions, NF-κB is sequestered in the cytoplasm in an inactive form through interaction with IκBα. Upon stimulation, IκBα is phos-

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† *Abbreviations*: NF-κB, nuclear factor-κB; MG132, carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal; LLnL, *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal; PDTC, pyrrolidine dithiocarbamate; ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; EMSA, electrophoretic mobility shift assay; and IAP, inhibitor of apoptosis protein.

phorylated, ubiquitinated, and then degraded by the proteasome, allowing NF-κB to translocate to the nucleus and initiate transcription [14]. Thus, in the system in which NF-κB may protect against cell death, prevention of IκBα degradation by proteasome inhibitors leads to suppression of NF-κB activation, which results in potentiation of apoptosis.

Although there have been increasing numbers of reports demonstrating pro- or antiapoptotic effects of proteasome inhibitors [5–8], their effects on apoptosis and NF-κB activation in human leukemia cells have not been investigated fully. This fact prompted us to examine the effects of proteasome inhibitors on apoptosis and NF-κB activation induced by etoposide in a human leukemia cell line and in fresh leukemia blasts. We found that proteasome inhibitors prevent apoptosis induced by etoposide in a human leukemic cell line, but not in fresh leukemia blasts, although NF-κB activation is inhibited in both cell types equally. These results suggest that the role of NF-κB in etoposide-induced apoptosis may be diverse depending on the cell type, at least among human leukemia cells.

MATERIALS AND METHODS

Reagents

Etoposide and MG132 were purchased from Calbiochem. LLnL and PDTC were obtained from Nacalai Tesque. The peptide SN50 was purchased from Biomol.

Cell Culture

A human myeloid leukemic cell line, U937, was provided by the Japanese Cancer Resources Bank. U937 cells were grown in RPMI 1640 medium supplemented with heat-inactivated 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/L), and streptomycin (100 μg/mL). The culture was maintained at 37° in a humidified atmosphere of 5% CO_2/95% air.

Isolation of Leukemia Blasts from Patients

All patients were newly diagnosed as having ALL or AML without any prior therapy. The characteristics of these patients are described briefly in Table 1. Freshly isolated peripheral blood obtained with informed consent was fractionated by Ficoll–Hypaque sedimentation. Then mononuclear cells were suspended immediately at a concentration of 1×10^6 cells/mL in RPMI 1640 medium supplemented with FBS, L-glutamine (2 mM), and antibiotics, and cultured as described earlier. Cell viability was determined by the trypan blue exclusion test, and exceeded 95% after the isolation procedure. The percentages of leukemia blasts were more than 95% in all cases.

Determination of Morphologically Apoptotic Cells

U937 cells and isolated leukemia blasts were incubated with various combinations of drugs described below for the indicated times. Then the cells were placed in a cytospin centrifuge and centrifuged onto a glass slide. Apoptotic cells were defined as those with condensed and fragmented nuclei in May–Giemsa-stained preparations [15].

Detection of Apoptotic Cells by Flow Cytometry

Apoptosis can be detected by measuring the sub-G_1 population with flow cytometry. Cells were fixed in 70% ethanol and treated with 10 μg/mL of RNase. They were stained with 25 μg/mL of propidium iodide and then subjected to analysis on a FACSCalibur flow cytometer with Cellquest software (Becton Dickinson) [16].

Preparation of Nuclear Extracts

Nuclear extracts were prepared according to a method described by Li *et al.* [17] with slight modifications. Cells (5×10^7) were harvested and washed once with PBS and once with 200 μL of ice-cold buffer A (10 mM HEPES–NaOH, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, pH 7.9). The cells were lysed in 200 μL of buffer A by gently passing the cell suspension through a 27-gauge needle. The nuclei then were collected by centrifuging for 8 sec in an Eppendorf microcentrifuge at 12,000 rpm and washed twice with buffer A. Crude nuclei were extracted with ice-cold buffer B (20 mM HEPES–NaOH, 25% glycerol, 420 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9) for 30 min on ice. Buffer C (100 μL) (20 mM HEPES–NaOH, 20% glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.9) was added, and the mixture was centrifuged for 15 min. Supernatants were collected, and the protein concentration was determined by using a Bio-Rad protein assay kit (Bio-Rad Laboratories).

Electrophoretic Mobility Shift Assay (EMSA)

A 26-bp synthetic oligonucleotide (5′-GATCCA-GAGGGGACTTTCCGAGAGGC-3′) containing the NF-κB consensus sequence (GGGGACTTTCC) was labeled with [γ-^{32}P]dGTP and Klenow enzyme. 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 10,000 cpm probe, 2 μg poly(dIdC), and 3 μg BSA. 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 electrophore-

sis, the dried gel was autoradiographed. For the competition assay, unlabeled NF-κB oligonucleotide was added at a 100-fold excess compared with the end-labeled fragment [15].

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 phenylmethysulfonyl fluoride, 1 μg/mL of aprotinin, and 1 μg/mL of leupeptin] at 4° with sonication. Protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad). Then SDS–sample buffer [65 mM Tris–HCl (pH 6.7), 3.3% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.2% bromophenol blue] was added to each 40 μg of postlysate protein, which was boiled for 3 min and electrophoresed on a 12% SDS–polyacrylamide gel. The proteins were transferred electrophoretically from the gel onto an Immobilon-P polyvinylidene difluoride membrane (Millipore). After blocking with 5% nonfat dry milk in TBST (10 mM Tris, 150 mM NaCl, 0.07% Tween 20), blots were incubated with rabbit anti-procaspase-3 antibody (PharMingen), anti-IκBα antibody (Santa Cruz), or antibody against IκBα phosphorylated at Ser32 (New England BioLabs), followed by anti-rabbit horseradish peroxidase-conjugated IgG (Santa Cruz), with three washings after each incubation. The proteins were visualized by the ECL chemiluminescent detection system (Amersham) [18].

RESULTS

Inhibition of Etoposide-Induced Apoptosis and Caspase-3 Activation by MG132 and LLnL in U937 Cells

We first examined the appearance of morphologically apoptotic cells following incubation with etoposide in the presence or absence of MG132 (10 μM) or LLnL (25 μM) using a human leukemia cell line, U937. As shown in Fig. 1A, cells with apoptotic features became evident 2 hr after starting incubation with 10 μg/mL of etoposide, and the proportion of apoptotic cells reached 50% after 6 hr. Pretreatment with MG132 or LLnL for 30 min clearly suppressed apoptotic changes for up to 6 hr. Under the conditions we employed, MG132 or LLnL alone did not induce any significant apoptosis. The inhibitory effects of these agents on etoposide-induced apoptosis were concentration-dependent (Fig. 1B). Concentrations of 10 μM for MG132 and 25 μM for LLnL were found to be the minimum concentrations for exerting the maximum inhibitory effects against apoptosis. Flow cytometry showed that treatment of U937 cells with etoposide for 6 hr increased the sub-G_1 population to approximately 60%. MG132 and LLnL obviously inhibited the increase of the sub-G_1 population, confirming the results of morphological apoptosis (Fig. 1C). Activation of an effector caspase is essential for execution of apoptosis and requires proteolysis of a proenzyme to an active fragment [1]. Western blotting revealed processing of procaspase-3 to a p17 fragment after 4 hr of incubation with etoposide. Consistent with the observations of morphological and flow cytometric studies, preincubation with MG132 or LLnL evidently blocked the cleavage of procaspase-3 (Fig. 1D). Again, these inhibitors did not activate caspase-3 by themselves. Such results suggest that MG132 or LLnL may inhibit an event upstream of caspase-3 activation.

Inhibition of Etoposide-Induced NF-κB Activation by MG132 and LLnL in U937 Cells

In previous studies, we and others [15, 18, 19] have demonstrated that NF-κB activation occurs during drug-induced apoptosis. Thus, we tested the effects of the proteasome inhibitors on NF-κB activation. As depicted in Fig. 2, the DNA-binding activity of NF-κB became detectable within 2 hr after treatment with 10 μg/mL of etoposide and was blocked by the addition of unlabeled competitors. Such NF-κB activation was found to be down-regulated at 4 hr of incubation. MG132 or LLnL prevented NF-κB activation almost completely during 4 hr of incubation.

Effects of MG132 and LLnL on the Degradation and Phosphorylation of IκBα in U937 Cells

Since NF-κB is activated through the degradation of IκBα mainly by the proteasome following phosphorylation and ubiquitination of IκBα [14], we tried to determine whether such a mechanism may be responsible for etoposide-induced NF-κB activation. Treatment of U937 cells with etoposide produced significant proteolysis of IκBα. Preincubation with MG132 or LLnL apparently prevented IκBα processing (Fig. 3A). Phosphorylation of IκBα at Ser32 became evident within 20 min following incubation with etoposide. Neither MG132 nor LLnL was able to block the phosphorylation of IκBα (Fig. 3B). These results exclude the possibility that MG132 or LLnL may inhibit an event upstream of IκBα phosphorylation.

Effects of Other NF-κB Inhibitors on Etoposide-Induced Apoptosis in U937 Cells

We also examined the effects of two agents that have been reported to prevent NF-κB activation through mechanisms other than proteasome inhibition. PDTC, an antioxidant, almost completely blocked etoposide-induced apoptosis in U937 cells. SN50 is a cell-perme-

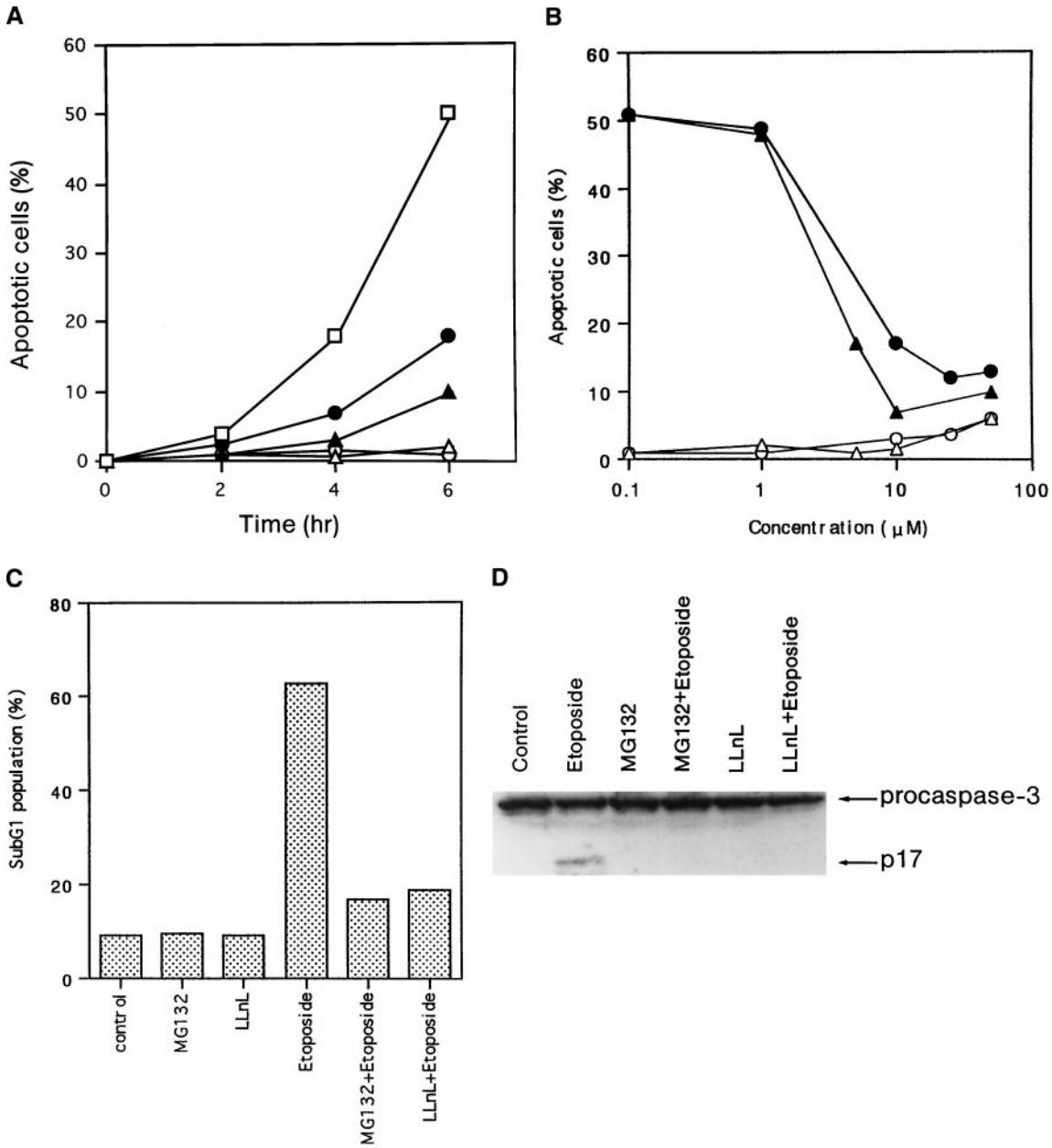

FIG. 1. Effects of proteasome inhibitors on apoptosis and caspase-3 activation. U937 cells were preincubated with or without 10 μM MG132 or 25 μM LLnL for 30 min. Then the cells were cultivated further in the presence or absence of 10 μg/mL of etoposide for the indicated intervals. (A) The proportion of morphologically apoptotic cells was assessed as described in Materials and Methods. The data represent the means of three separate experiments. Key: (□) etoposide; (▲) MG132 plus etoposide; (●) LLnL plus etoposide; (△) MG132; and (○ LLnL. (B) Concentration-dependent effects of MG132 and LLnL on etoposide-induced apoptosis. Key: (▲) MG132 plus etoposide; (●) LLnL plus etoposide; (△) MG132; and (○) LLnL. (C) Detection of apoptosis by measuring the sub-G_1 population with flow cytometry. U937 cells were treated with 10 μg/mL of etoposide for 6 hr with or without pretreatment with the proteasome inhibitors. Then cells were stained with propidium iodide, and the proportion of sub-G_1 cells was analyzed by a flow cytometer. Data represent the means of three separate experiments. (D) Western blot analysis of procaspase-3 and a p17 active fragment at 4 hr after addition of etoposide.

able peptide that contains the nuclear localization sequence of NF-κB, thereby inhibiting translocation of activated NF-κB into the nucleus [20]. Pretreatment with 100 μg/mL of SN50 clearly reduced the appearance of apoptotic cells (Fig. 4). These results suggest that prevention of NF-κB activation may be associated with suppression of etoposide-induced apoptosis in U937 cells.

Effects of MG132 and LLnL on Morphological Apoptosis and NF-κB Activation in Leukemia Blasts Isolated from Patients

As depicted in Table 1, more than 50% of isolated leukemia cells from three ALL and three AML patients underwent apoptosis when cultured with 10 μg/mL of etoposide for 12 hr. In sharp contrast with the results in U937, however,

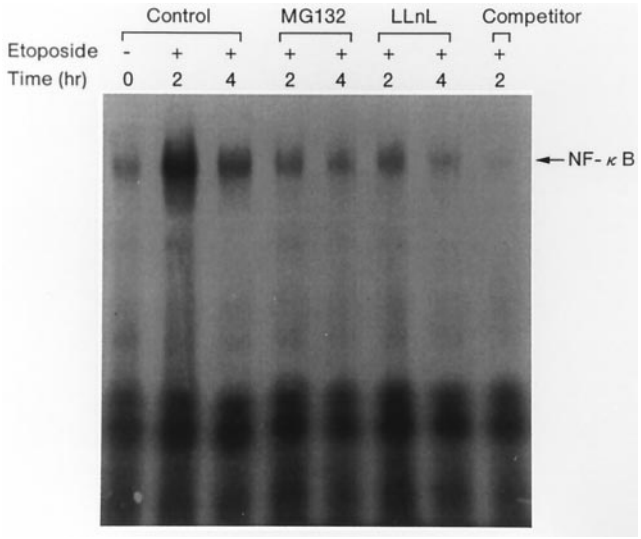

FIG. 2. Effects of proteasome inhibitors on NF-κB activation. U937 cells were cultured with 10 μg/mL of etoposide with or without pretreatment with 10 μM MG132 or 25 μM LLnL. Cells then were taken at the indicated times and subjected to EMSA for NF-κB mobilization.

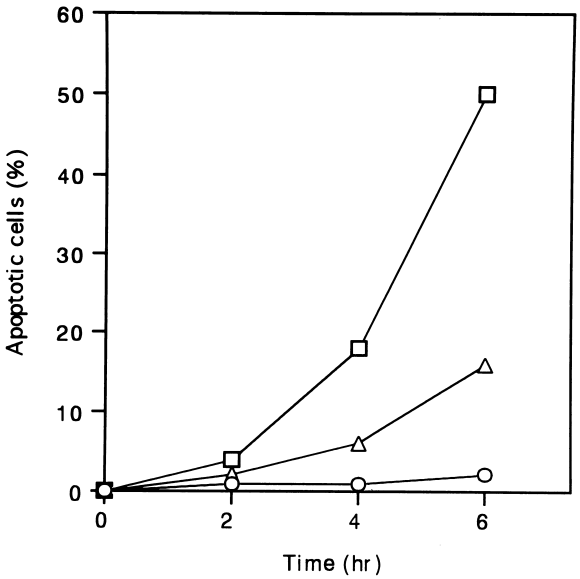

FIG. 4. Effects of PDTC and SN50 on etoposide-induced morphological apoptosis in U937 cells. Cells were incubated with 10 μg/mL of etoposide with or without pretreatment with 100 μM PDTC or 100 μg/mL of SN50. Key: (□) etoposide; (△) SN50 plus etoposide; and (○) PDTC plus etoposide. Data represent the means of three separate experiments.

pretreatment with MG132 or LLnL failed to inhibit etoposide-induced apoptosis. Notably, significant apoptosis was observed in these cells following incubation with proteasome inhibitors alone. EMSA revealed that etoposide induced NF-κB activation in leukemia blasts 4 hr after the start of the culture. Coincubation with proteasome inhibitors clearly suppressed etoposide-induced NF-κB activation (Fig. 5).

DISCUSSION

In the present study, we demonstrated that the proteasome inhibitors MG132 and LLnL prevented etoposide-induced morphological apoptosis and caspase-3 activation in a human leukemia cell line, U937. Such effects were also seen when we used HL-60, another human leukemia cell line, as target cells (Watanabe K and Kubota M, unpublished observation). In contrast, these agents were unable to inhibit etoposide-induced apoptosis in freshly isolated leukemia blasts from patients with ALL or AML. Proteasome inhibitors alone, however, were found to induce apoptosis in these cells. At lower concentrations of MG132 (less than 1.0 μM) or LLnL (2.5 μM) where these drugs alone did not induce significant apoptosis, they neither inhibited nor augmented etoposide-induced apoptosis in leukemia blasts (data not shown). Therefore, it is difficult to discuss the synergy between etoposide and proteasome inhibitors in fresh leukemia blasts. In spite of their different effects on apoptosis, MG132 and LLnL prevented NF-κB activation in both leukemia cell lines and leukemia blasts. Although previous investigators have suggested that inhibition of NF-κB potentiates chemosensitivity [21], our data suggest that NF-κB may have an apoptosis-promoting function in leukemia cell lines, but not in fresh leukemia blasts.

A large body of recent investigations has indicated an

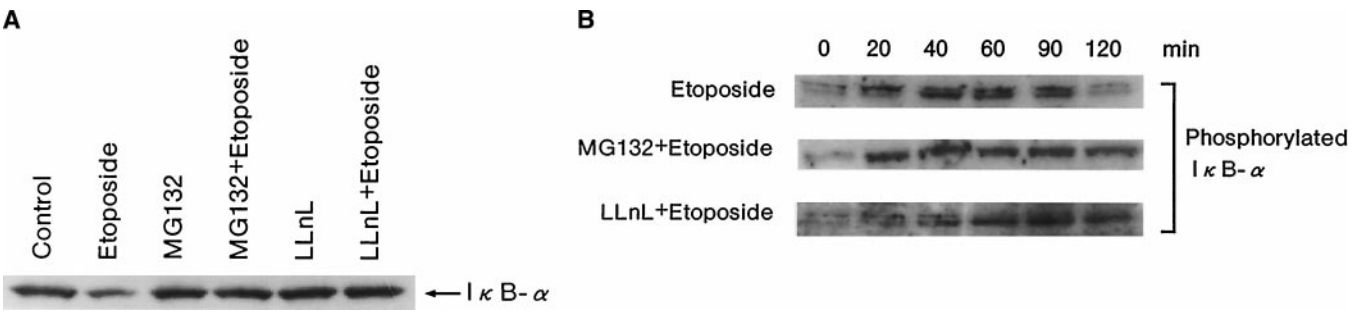

FIG. 3. Effects of proteasome inhibitors on degradation and phosphorylation of IκBα. (A) Western blot analysis of IκBα. Cells were treated with or without 10 μg/mL of etoposide for 4 hr with or without pretreatment with 10 μM MG132 or 25 μM LLnL. Cell extracts were prepared for western blotting using anti-IκBα-antibody. (B) Western blot analysis of phosphorylated IκBα. Cells were treated with 10 μg/mL of etoposide for the indicated intervals with or without pretreatment with 10 μM MG132 or 25 μM LLnL. A western blot was carried out using an antibody to IκBα phosphorylated at Ser32.

TABLE 1. Percentage of morphologically apoptotic cells in freshly isolated leukemia cells

		Apoptotic cells (%)					
		Control			Etoposide		
Diagnosis	Age	Control	MG132	LLnL	Control	MG132	LLnL
1. Early preB ALL	8 months	22.3	48.0	79.7	78.3	94.0	92.5
2. PreB All	2 years	6.0	48.5	55.0	63.0	87.5	89.5
3. T-ALL	14 years	5.5	82.3	78.5	67.3	95.7	91.0
4. AML M1	3 years	9.7	47.3	49.7	57.3	80.0	74.7
5. AML M4	1 year	5.0	32.3	31.5	63.0	75.5	82.5
6. AML M5	14 years	4.5	48.5	55.5	67.5	78.5	82.0

Freshly isolated acute leukemia cells were cultivated for 12 hr under the conditions described above. Then apoptotic cells were determined as described in Materials and Methods. Data represent the means of triplicate experiments. The concentration of each drug was: MG132, 10μM; LLnL, 25 μM; and etoposide, 10 μg/mL.

antiapoptotic role of NF-κB activation (reviewed in Ref. 13). However, several reports of evidence against this notion also have been presented. For example, inhibition of NF-κB activation by either parthenolide or a transdominant negative form of IκBα prevents hydrogen peroxide-induced apoptosis [22]; overexpression of c-Rel, a member of the NF-κB family, leads to death in avian bone marrow cells [23]; the induction of apoptosis by serum withdrawal is associated with NF-κB activation, which can be blocked by overexpression of a transdominant negative NF-κB p65 (RelA) subunit [24]; inhibition of NF-κB with a transcriptional factor decoy rescues the cells from Sindbis virus-induced death [25]. Moreover, DNA-damaging agents including etoposide induce Fas ligand expression via NF-κB activation and subsequent apoptosis in T lymphocytes [26]. β-Lapachone concomitantly suppresses tumor necrosis factor-induced apoptosis and NF-κB activation in U937 cells [27]. One possible explanation for the different role of NF-κB may lie in the differences of inducers and target cells. It is speculated that NF-κB may regulate apoptosis by the induction of genes encoding apoptosis-promoting or -inhibiting factors. For example, cIAP-1 and 2, members of the IAP family, were recently identified as NF-κB-dependent factors that inhibit activation of caspases [28, 29]. NF-κB possibly protects cells against apoptosis through activation of such an antiapoptotic factor. Those cells in which NF-κB appears to promote cell death, such as the leukemia cell lines used in this study, might be defective in antiapoptotic factor(s), resulting in the predominance of apoptosis-promoting signals to induce cell death. Although

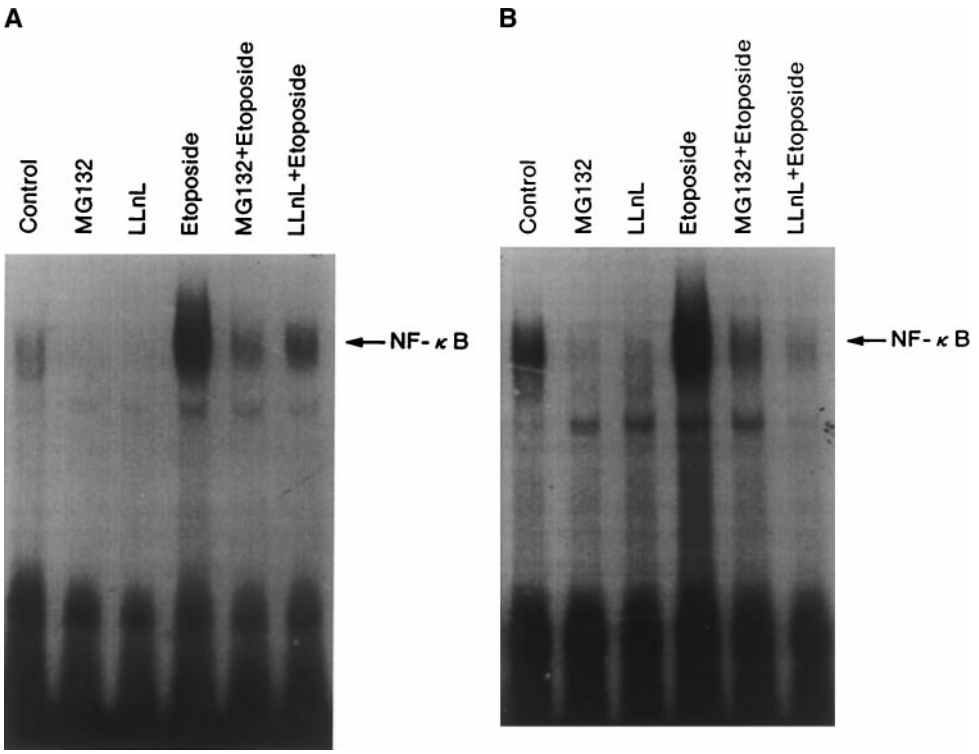

FIG. 5. Effects of proteasome inhibitors on NF-κB activation in leukemia blasts from (A) patient 3 and (B) patient 4. Isolated leukemia blasts were cultured with 10 μg/mL of etoposide with or without pretreatment with 10 μM MG132 or 25 μM LLnL. Cells were taken at 4 hr and subjected to EMSA for NF-κB mobilization.

both cIAP-1 and -2 were detected by western blotting in untreated U937 cells, the expression of these proteins did not change significantly after 6 hr of treatment with etoposide (data not shown). A role of other antiapoptotic genes regulated by NF-κB in this system remains to be determined.

Proteasome inhibitors have been shown to induce apoptosis in rapidly dividing, transformed cells [5, 6], whereas they seem to prevent apoptosis in neuronal cells [7] or thymocytes [8]. Here, we demonstrated that proteasome inhibitors can induce apoptosis in fresh leukemia cells from patients. As far as we know, there exists only one report showing induction of apoptosis by proteasome inhibitors in fresh leukemia cells, i.e. chronic lymphocytic leukemia lymphocytes [11]. Diverse functions of proteasome inhibitors have been reported (reviewed in Ref. 4). We have shown that MG132 and LLnL can inhibit IκBα degradation without preventing IκBα phosphorylation in U937 cells. This fact indicates that the site of action of these agents in inhibiting apoptosis induced by etoposide is downstream from IκBα phosphorylation. Besides IκBα, the proteasome is known to digest various cellular proteins, such as p53 [30] and Jun [31], that are involved in the control of cell cycle and survival. An association of p53 in our system is quite unlikely, at least in leukemia cell lines, because both U937 and HL-60 cells are reported to be deficient in p53 [32]. Moreover, the fact that other NF-κB inhibitors such as PDTC and SN50 also inhibit etoposide-induced apoptosis in U937 cells suggests that the target molecule downstream of the proteasome inhibition is likely to be the IκBα/NF-κB system.

NF-κB has now emerged as one of the new targets for cancer chemotherapy, based on the notion that prevention of the antiapoptotic function of NF-κB may result in the sensitization of cells to anticancer drugs [13, 33]. However, researchers should be cautious, since inhibition of NF-κB may have an antiapoptotic function, as indicated in the present study. To clarify the molecular events occurring downstream of NF-κB activation during apoptosis in each cell type may become important when chemotherapy targeting NF-κB is brought into clinical settings.

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