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Cepharanthine Activates Caspases and Induces Apoptosis in Jurkat and K562 Human Leukemia Cell Lines

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Abstract Cepharanthine (CEP) is a known membrane stabilizer that has been widely used in Japan for the treatment of several disorders such as anticancer therapy-provoked leukopenia. We here report that apoptosis was induced by low concentrations (1–5 μM) of CEP in a human leukemia T cell line, Jurkat, and by slightly higher concentrations (5–10 μM) in a human chronic myelogenous leukemia (CML) cell line K562, which expresses a p210 antiapoptotic Bcr-Abl fusion protein. Induction of apoptosis was confirmed in both Jurkat and K562 cells by DNA fragmentation and typical apoptotic nuclear change, which were preceded by disruption of mitochondrial membrane potential and were induced through a Fas-independent pathway. CEP treatment induced activation of caspase-9 and -3 accompanied by cleavage of PARP, Bid, lamin B_1, and DFF45/ICAD in both Jurkat and K562 cells, whereas caspase-8 activation and Akt cleavage were observed only in Jurkat cells. The CEP-induced apoptosis was completely blocked by zVAD-fmk, a broad caspase inhibitor. Interestingly, CEP treatment induced remarkable degradation of the Bcr-Abl protein in K562 cells, and this degradation was prevented partially by zVAD-fmk. When used in combination with a nontoxic concentration of herbimycin A, lower concentrations (2–5 μM) of CEP induced obvious apoptosis in K562 cells with rapid degradation or decrease in the amount of Bcr-Abl and Akt proteins. Our results suggest that CEP, which does not have bone marrow toxicity, may possess therapeutic potential against human leukemias, including CML, which is resistant to anticancer drugs and radiotherapy. J. Cell. Biochem. 82: 200–214, 2001.

Key words: cepharanthine; apoptosis; caspase; human leukemia cells; Bcr-Abl; Fas; herbimycin A; signal transduction

Apoptosis (programmed cell death) ensures the homeostasis of tissues during development, host defense, and aging. Aberrant cell survival due to insufficient apoptosis, however, may be linked to the development and/or progression of human malignancies, and a number of agents for cancer chemotherapy primarily act through induction of apoptosis in target cells [Glinsky, 1997; Hannun, 1997].

Caspases, currently known as the central executioners of many, if not all, apoptotic pathways, are cysteine proteases that cleave substrates after aspartate residues [Cohen, 1997; Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997], and at least 14 different subtypes have been identified in mammalian cells. A caspase is first synthesized as an inactive tetrameric complex composed of two heterodimeric subunits of about 10 and 20 kD, and caspases are activated through autoproteolysis of the precursor or through mutual processing to form a cascade [Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997]. An increasing number of proteins have been found to be cleaved by caspases, including poly (ADP-ribose) polymerase (PARP), Akt, Bid, lamins, and DNA fragmentation factor 45 (DFF45) / inhibitor of caspase-activated DNase (ICAD) [Lazebnik et al., 1994; Neamati et al., 1995; Liu et al., 1997; Enari et al., 1998; Li et al., 1998; Luo et al., 1998; Rokudai et al., 2000]. After the cleavage of DFF45/ICAD, caspase-activated DNase (CAD) enters the nucleus and leads to

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fragmentation of genomic DNA into nucleosomal fragments, a hallmark of apoptosis [Enari et al., 1998; Sakahira et al., 1998] .

Two pathways of caspase activation for apoptosis induction have been identified. The first one starts at death receptors such as Fas [Nagata, 1997]. When a Fas ligand binds to the Fas death receptor, the adaptor molecule FADD/Mort-1 becomes recruited to the receptor, allowing binding and proteolysis of procaspase-8 for generation of activated caspase-8 [Medema et al., 1997; Scaffidi et al., 1997]. The activated caspase-8 processes effector caspases (caspase-3, -6, and -7) for activation [Nagata, 1997; Nicholson and Thornberry, 1997]. In the second pathway, diverse proapoptotic signals converge at the mitochondrial level, inducing the translocation of cytochrome c (cyt. c) and apoptosis-inducing factor (AIF) from mitochondria to the cytoplasm, thereby triggering the cascade of caspase activation (cyt. c) or entering the nucleus directly (AIF) for the induction of DNA fragmentation [Green and Reed, 1998; Susin et al., 1999]. In addition, the two pathways may be connected in such a way that activated caspase-8 cleaves Bid and truncated Bid (tBid) acts on the mitochondria to induce the release of cyt. c [Li et al., 1998; Luo et al., 1998], thus inducing activation of the downstream caspases.

Cepharanthine (CEP), a biscoclaurine alkaloid extracted from Stephania cepharantha Hayata [Tomita et al., 1967], is known as a membrane-interacting agent that has membrane-stabilizing activity [Utsumi et al., 1976] and has been widely used in the treatment of snake venom-induced hemolysis, nasal allergy, and leukopenia induced by anticancer drugs and radiation therapy in Japan [Kato and Suzumura, 1987]. It has been reported that CEP potentiates the activity of some antitumor agents and restores the effect of anticancer drugs in multidrug-resistant cancer cells possibly through perturbing plasma membrane function for increasing intracellular accumulation of anticancer drugs [Kato and Suzumura, 1987; Nagaoka et al., 1987; Shiraishi et al., 1987]. Some recent reports have provided evidence that CEP itself displays some direct antitumor effect, inhibiting the growth of Ehrlich ascites tumor [Asaumi et al., 1995] and inducing apoptosis in murine leukemia cells [Furusawa et al., 1998]. However, nothing is known about the molecular mechanism of the direct antitumor effect of CEP or the signal transduction pathways for apoptosis induction by CEP. Moreover, there have been few reports on the antitumor effects of CEP on human leukemia cells.

In our current study, we examined the antitumor effects of CEP on two human leukemia cell lines, Jurkat and K562, for apoptosis induction. K562 is a chronic myelogeneous leukemia (CML) cell line that is cytologically characterized by the presence of the Philadelphia (Ph) chromosome resulting from a reciprocal translocation between chromosome 9 and 22 [Kurzrock et al., 1988]. This translocation, which produces the hybrid *bcr-abl* gene, results in the synthesis of a chimeric Bcr-Abl protein that expresses an elevated level of Abl tyrosine kinase activity [Konopka et al., 1984]. The *bcr-abl* gene has been reported to play a significant role in the generation of CML [McLaughlin et al., 1987]. The K562 cell line was derived from a CML patient and was found to express p210 Bcr-Abl protein [Lozzio and Lozzio, 1975]. Previous studies have shown that this cell line is particularly resistant to cell death via apoptosis, irrespective of stimuli [McGahon et al., 1994; Ray et al., 1996]. Based on the biological characteristics of K562 cells, we also investigated the role of the Bcr-Abl protein in CEP-induced apoptosis.

We here report that CEP triggers apoptosis in both human Jurkat and K562 cells mainly via a mitochondrial signal pathway and that Bcr-Abl oncoprotein is degraded during CEP-induced apoptosis in K562 cells. Taken together, the results suggest that CEP might be a new drug candidate for the treatment of human leukemias. Particularly for CML therapy, CEP in combination with herbimycin A (HMA) [Uehara et al., 1989], a selective tyrosine kinase inhibitor that promotes the degradation or reduction in the amount of Bcr-Abl and Akt proteins, may be more effective.

MATERIALS AND METHODS

Chemical Reagents

Purified CEP was purchased from WAKO Biochemicals (Osaka, Japan). A stock solution (20 mM) in dimethyl sulfoxide (DMSO) was conserved at $-80^{\circ}C$ for not more than three months. Further dilutions were made in a culture medium just before use. The final concentration of DMSO in the culture was kept

at $\leq$0.2%, a concentration that was nontoxic to the cells. Herbimycin A, 3,3-dihexyloxacarbocyanine iodide ($DiOC_6$) and 4,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma (St. Louis, MO), and zVAD-fmk [Zhu et al., 1995] was obtained from Calbiochem (Bad Soden, Germany).

Cell Lines, Cell Culture, and Drug Treatment

Jurkat and K562 cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The cells were grown in RPMI-1640 (Nissui Pharmaceutical Co., Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For all experiments, 1.0×10^6 /well of logarithmically growing cells were placed in each well of a 6-well plate containing 2 ml RPMI-1640 + 10% FBS with or without CEP. DMSO ($\leq$0.2%) was added to all of the control cultures.

Antibodies

The monoclonal antibodies against caspase-8 (5F7) and DFF45/ICAD (6B8), and cytotoxic monoclonal anti-Fas IgM (CH-11) were purchased from Medical and Biological Laboratories, Co. (Nagoya, Japan). Anti-caspase-9 polyclonal antibody and anti-Abl monoclonal antibody (8E9) were obtained from Pharmigen (San Diego, CA), and anti-β-actin monoclonal antibody (AC15) was obtained from Sigma (St. Louis, MO). Anti-PARP polyclonal antibody was obtained from Upstate Biotechnology Incorporated (Lake Placid, NY), and anti-Akt polyclonal antibody was obtained from New England Biolabs (Beverly, MA). Anti-caspase-3 and anti-Bcl-$X_{S/L}$ polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Bid polyclonal antibody was purchased from Santa Cruz Biotechnology. Anti-lamin B_1 monoclonal antibody (101-B7) was obtained from Oncogene Research Products (Cambridge, MA), and anti-Fas blocking monoclonal antibody (SM1/23) was obtained from Bender MedSystems (Vienna, Austria).

Detection of Internucleosomal Fragmentation of Genomic DNA by Agarose Gel Electrophoresis

DNA fragmentation was detected as described previously [Herrmann et al., 1994; Dohi et al., 1996]. In brief, after incubation with the designated concentrations of the chemicals, cells were pelleted by centrifugation. One hundred microliter of lysis buffer containing 10 mM Tris-Hcl (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100 was added to the pellet, and the preparation was left at 4°C for 15 min. After centrifugation at 15,000 rpm for 20 min at 4°C, the supernatant was incubated at 37°C with 0.8 mg/ml of RNase A for 1 h followed by additional incubation for 1 h with 0.8 mg/ml of proteinase K. Fragments of DNA were then precipitated with 20 μl of 5M NaCl and 120 μl of 2-propanol and left overnight at −20°C. Following centrifugation for 20 min, DNA fragmentation was analyzed by using 1.4% agarose gel electrophoresis at 50 V for 1.5 h.

Morphological Change of Apoptotic Cells as Determined by a DAPI Nuclear Staining Assay

After treatment with or without CEP, cells were washed once with PBS and then fixed with a methanol:acetic acid solution (3:1) for 30 min at room temperature. After fixation, the cells were washed twice with PBS, and the cell pellets were harvested. Twenty microliter of PBS was added and then mixed with 4 μl of 10 μg/ml of DAPI. After 30 min of staining, the cell samples were placed on glass slides and covered with cover glasses. Apoptotic chromatin condensation and segregation were observed under a fluorescence microscope (Olympus BX60, Japan, 100×) within 30 min.

Flow Cytometric Analysis of Mitochondria Membrane Potential ($\Delta\psi_m$) Disruption

Mitochondria membrane potential analysis was performed basically as described previously [Marzo et al., 1998]. In brief, cells were collected and washed twice with PBS. The cell pellet was incubated in 500 μl of PBS containing 50 nM $DiOC_6$ for 30 min. This $DiOC_6$ cyanine dye accumulates in the mitochondria matrix under the influence of the $\Delta\psi_m$. $DiOC_6$ membrane potential-related fluorescence was analyzed using a FACScalibur flow cytometer.

Quantitative Measurement of Apoptosis by Flow Cytometry

Analysis of apoptosis percentage was performed as previously described with slight modifications [Dong et al., 1998]. Briefly, after incubation for the indicated times, cells were harvested and fixed in 70% ethanol for 1 h on ice. The fixed cells were washed twice with PBS and

suspended in 50 μl PBS. An 8-μl aliquot of 10 mg/ml RNase A solution was added, and the mixture was incubated for 1 h at 37°C. Cells were stained in PI solution (50 μg/ml in 0.1% sodium citrate, 0.1% NP-40) and incubated for 15 min on ice. Apoptosis was scored by the percentage of cells appearing in the area below the G_1/G_0.

Immunoblot Analysis

SDS-PAGE and immunoblot analysis were performed as previously described [Nakashima et al., 1991]. Briefly, cells were washed in cold PBS and lysed in sample buffer (2X: 62.5 mM Tris-Hcl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride PMSF). Subsequently, appropriate protein amounts (20 μg) were separated under reducing conditions by 8 to 15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Eschborn, Germany). The blots were blocked in 5% nonfat milk or 3% BSA overnight at 4°C with gentle shaking. This was followed by incubation with the respective antibody for 3 h at room temperature and then with anti-rabbit, anti-mouse, or anti-goat peroxidase-conjugated secondary IgG antibodies (Tago, Burlingame, CA). Immune complexes were detected using an enhanced chemiluminescence detection method by immersing the blot for 1 min in a 1:1 mixture of chemiluminescence reagents A and B (DuPont Boston, MA). Finally, the membranes were exposed to Fujifilm for an appropriate time to obtain clear bands. In all immunoblotting experiments, the blots were re-probed with an anti-β-actin antibody to control for protein loading.

Reproduction of the Experiments

At least three independent experiments were performed for each assay. Representative results of the experiments are shown in each figure.

RESULTS

CEP Induces Apoptosis With Typical DNA Fragmentation in Jurkat and K562 Leukemia Cells

We first investigated whether CEP induces apoptosis in Jurkat and K562 cells. When analyzed by agarose gel electrophoresis, CEP dose-dependently and time-dependently induced DNA fragmentation in both cell lines (Fig. 1). In Jurkat cells, CEP at a dose as low as 1 μM induced visible DNA fragmentation after 20 h of incubation, and internucleosomal DNA fragmentation was most remarkable at 10 μM of CEP (Fig. 1A, left). On the other hand, CEP at a concentration of 5 μM or higher induced visible DNA fragmentation in K562 cells after 20 h of incubation, and the most obvious DNA fragmentation was observed at 20 μM of CEP (Fig. 1A, right). When the concentration of CEP was increased above 10 (in Jurkat cells) or 20 μM (in K562 cells), CEP resulted in gradually less DNA fragmentation (Fig. 1A). At 100 μM of CEP, no DNA fragmentation was detected in either of the two cell lines (Fig. 1A), although most of the cells died, which was confirmed by trypan blue exclusion assay (data not shown). This suggested that upon treatment with CEP at a high concentration, Jurkat and K562 cell death may also be mediated by a non-

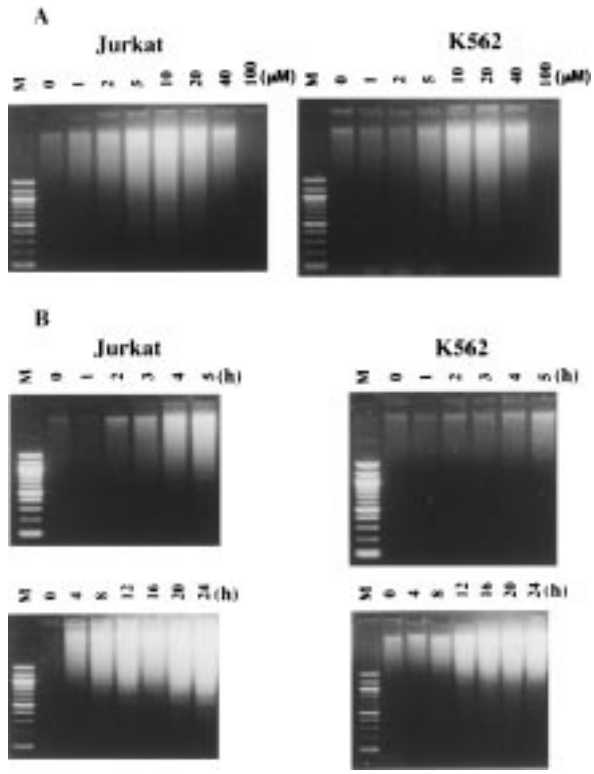

Fig. 1. CEP induces DNA fragmentation in Jurkat and K562 cells. Jurkat and K562 cells were treated with various concentrations of CEP and incubated for 20 h **(A)** or with 10 μM of CEP for the indicated times **(B)**. Internucleosomal DNA fragmentation was analyzed by agrose gel electrophoresis. One hundred-basepairs DNA ladder size markers (M) have been put on the left end lane of each gel.

apoptotic mechanism. In the time course test (Fig. 1B), the initial DNA fragmentation was observed as early as 2 h after exposure to 10 μM of CEP in Jurkat cells but only after 5 h in K562 cells. CEP-induced DNA fragmentation thus occurred more slowly in K562 cells than in Jurkat cells, although once started, DNA fragmentation continued to progress for at least 24 h in both of the cell lines. These results demonstrated that K562 cells exhibit some resistance to CEP-induced apoptosis.

To examine the morphological changes during CEP-induced apoptosis, we stained the nuclei with DAPI, a chemical that binds to cellular DNA by forming a stable complex that excites fluorescence. As shown in Figure 2, after treatment with 10 μM of CEP for 12 (Jurkat cells) or 20 h (K562 cells), we observed many cells containing condensed and fragmented nuclei stained with DAPI (31/100 cell counts in Jurkat cells and 42/100 counts in K562 cells). Very few untreated control cells (2/100 cell counts in both of the cell lines) displayed such properties. These results indicated that CEP induces typical apoptosis in both of the cell lines.

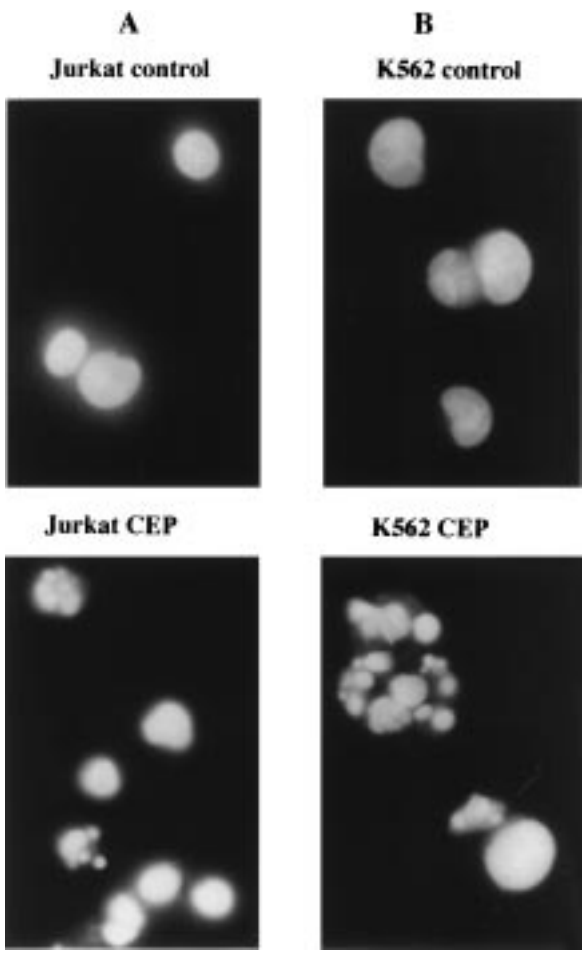

Fig. 2. CEP triggers apoptotic morphological changes in Jurkat and K562 cells. Jurkat **(A)** and K562 **(B)** cells were treated with 10 μM of CEP for 12 h (A) or for 20 h (B). Nuclear staining assay with DAPI was performed, and nuclear morphological changes were observed under a fluorescence microscope.

Disruption of Mitochondrial Membrane Potential in CEP-Induced Apoptosis

Disruption of mitochondrial membrane potential ($\Delta\psi_m$), which is observed at an early stage of apoptosis in many cells, results in opening of the permeability transition (PT) pores, causing a local disruption of the outer mitochondrial membrane [Petit et al., 1995; Zamzami et al., 1995]. As a consequence, the soluble intermembrane proteins, including cyt. c and AIF, are released, and these proteins may contribute to the activation of capases (cyt. c) or enter the nucleus directly (AIF) for chromatin condensation and DNA fragmentation [Susin et al., 1999]. Based on this theory, we next assessed the mitochondrial $\Delta\psi_m$ after CEP treatment by measuring mitochondrial uptake of a membrane potential-sensitive dye, $DiOC_6$, in Jurkat and K562 cells. When Jurkat cells were treated with 10 μM of CEP, the populations losing mitochondrial membrane potential were 13% at 4 h incubation and 24.8% at 8 h, whereas the percentages in K562 cells were 6.1% at 4 h and 13.6% at 8 h (Fig. 3). These results indicated that mitochondrial membrane impairment due to CEP treatment occurred more slowly and mildly in K562 cells than in Jurkat cells. This might be one factor that is responsible for the delayed and milder apoptosis in K562 cells. These observations also suggested that an apoptosis-inducing mechanism via mitochondria triggered by CEP operates in both Jurkat and K562 cell lines.

CEP-Induced Apoptosis is Fas-Independent

It has been proposed that induction of apoptosis by cytotoxic agents may involve ligand/receptor-driven amplifier systems such as the Fas system [Friesen et al., 1996]. Controversially, some reports have described the induction of Fas-independent apoptosis after treatment with cytotoxic drugs [Eischen et al., 1997; Wesselborg et al., 1999]. To investigate whether CEP-induced apoptosis is dependent on Fas/Fas ligand interaction, we

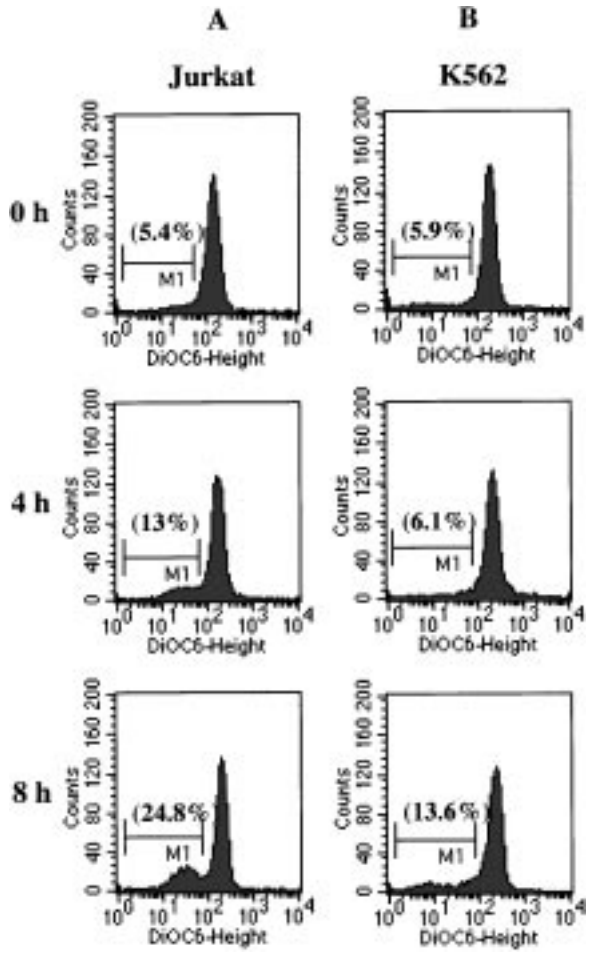

Fig. 3. CEP induces damage to the mitochondria membrane potential ($\Delta\psi_m$) in Jurkat and K562 cells. Jurkat **(A)** and K562 **(B)** cells were treated with 10 μM of CEP for the indicated periods and analyzed by FACScalibur after staining with $DiOC_6$. Numbers in parentheses represent percentages of cells with low $\Delta\psi_m$.

examined the apoptosis-inducing effect by incubating Jurkat cells with CEP in the presence or absence of an anti-Fas blocking antibody (SM1/23) [Liu et al., 2000]. As shown in Figure 4A and B, both CEP and cytotoxic anti-Fas antibody (CH-11) induced distinct apoptosis and caspase-8 activation in Jurkat cells. SM1/23 apparently prevented CH-11-induced apoptosis, whereas it had no inhibitory effect on CEP-induced apoptosis and caspase-8 activation (Fig. 4A and B). Accordingly, SM1/23 did not block CEP-induced caspase-9 and -3 activation, PARP and ICAD cleavage in Jurkat cells (data not shown). We also made comparative measurements of the expression levels of Fas and Fas ligand on CEP-treated and untreated Jurkat cells, and we found that the expression levels of both the Fas receptor and Fas ligand were not changed following CEP treatment (data not shown). Considering these results together with the finding that CEP induced remarkable apoptosis in K562 cells, which do not express functional Fas [Hampton and Orrennius, 1997; Boggs et al., 1998], we concluded that CEP-induced apoptosis is independent of the signal mediated by the Fas death receptor.

CEP-Induced Apoptosis is Caspase-Dependent

Caspases, a family of aspartate-specific cysteine proteases, play a pivotal role in the execution of programmed cell death [Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997]. To further analyze the CEP-induced pathway of apoptotic signal transduction, we performed a strict time course experiment from 4 to 24 h at intervals of 4 h by treating Jurkat and K562 cells with 10 μM of CEP. We tested the processing of procaspases by immunoblot analysis using antibodies specific to individual proteases. First, we investigated the processing of caspase-8, the most proximal caspase during death receptor-mediated apoptosis [Nagata, 1997]. Caspase-8 is synthesized as two isoforms of about 56/55 kD (caspase-8a and -8b), which, after formation of intermediate cleavage products of 43 and 41 kD, are processed into p18 and p10 heterodimers [Medema et al., 1997; Scaffidi et al., 1997]. When we examined the blots with an antibody directed against both the full-length caspase-8 and 43/41-kD intermediate cleaved forms (this antibody does not recognize p18 and p10 active forms), we found that CEP treatment resulted in cleavage of capase-8 into its characteristic intermediate fragments in Jurkat cells (Fig. 5A). The cleaved products were increased time-dependently for at least 24 h after CEP treatment. In contrast, we did not detect any cleaved products of caspase-8 even at 24 h after CEP treatment in K562 cells (Fig. 5B). We next tested for caspase-9, which is believed to transfer the death signal from mitochondria [Kuida, 2000]. As shown in Figure 5A and B, caspase-9 was cleaved to generate a 36-kD fragment and an 18-kD fragment in Jurkat cells. In K562 cells, the cleavage mainly produced a large 36-kD active form (Fig. 5B). Both occurred in a time-dependent manner. Finally, we checked the processing of caspase-3, which is considered to play a central role in many types of stimuli-induced apoptosis [Nicholson and Thornberry, 1997;

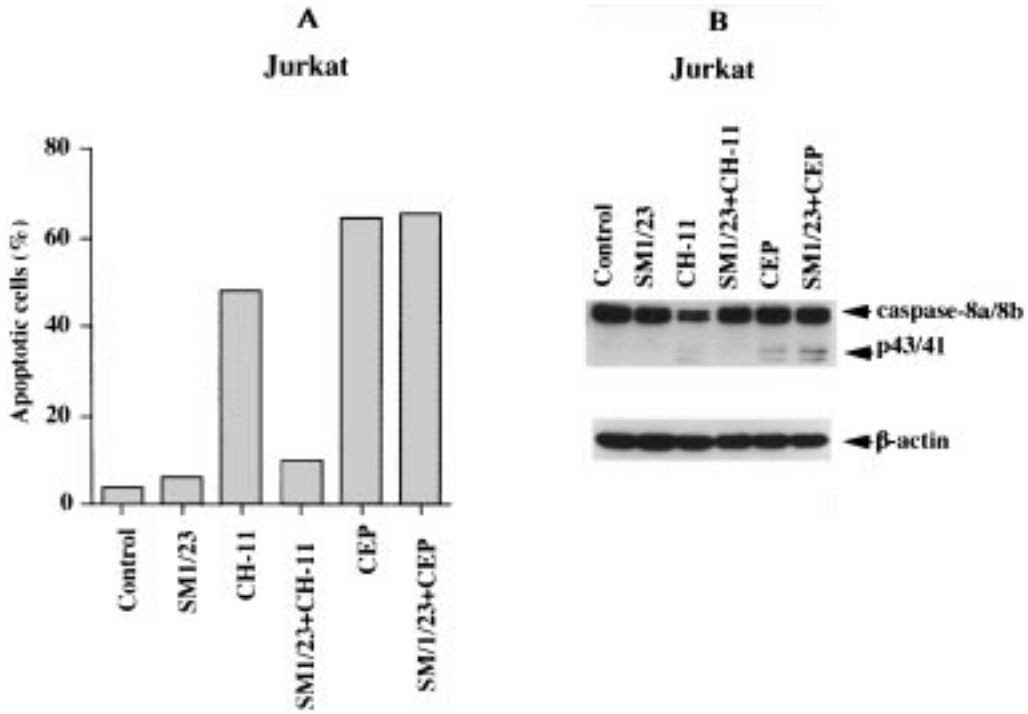

Fig. 4. CEP-induced apoptosis in Jurkat cells is not blocked by anti-Fas blocking antibody. Jurkat cells were preincubated with 1 μg/ml of anti-Fas blocking antibody (SM1/23) for 30 min followed by treatment with 10 μM of CEP or 200 ng/ml of cytotoxic anti-Fas antibody (CH-11) for 20 h. Quantitative measurement of apoptotic cells was performed by flow cytometry **(A)**, and processing of caspase-8 was detected by immunoblotting analysis **(B)**. β-actin was used as a control for protein loading.

Salvesen and Dixit, 1997]. Incubation of Jurkat and K562 cells with 10 μM of CEP for 4 to 24 h resulted in activation of caspase-3, as was evident by the appearance of a 17-kD proteolytic product of caspase-3 (dominant in Jurkat cells) or an 11-kD cleaved product (dominant in K562 cells) (Fig. 5A and B).

To determine more directly whether caspase activation is indispensable for CEP-induced apoptosis, a broad peptide inhibitor of caspases, zVAD-fmk, was used. As shown in Figure 5C and D, pretreatment of Jurkat and K562 cells with 100 μM of zVAD-fmk for 30 min completely inhibited CEP-induced DNA fragmentation, as determined by agarose gel electrophoresis analysis. Consistent results were obtained when the percentages of apoptotic cells were analyzed by flow cytometry (PI staining) (data not shown). These results confirmed that CEP-induced apoptosis in the two cell lines is caspase-dependent.

Cleavage Analyses of PARP, Bid, Akt, Lamin B_1, and DFF45/ICAD During CEP-Induced Apoptosis

During apoptosis, activation of capases leads to cleavage of many important cellular substrates, and this cleavage is generally believed to be responsible for acceleration of the apoptosis process [Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997]. PARP is one of the most commonly used indicators for activated cysteine proteases during apoptosis [Lazebnik et al., 1994]. Bid is a Bcl-2 member containing only a BH3 domain, and truncated Bid (tBid) is believed to be a potent apoptosis-inducing agent [Li et al., 1998; Luo et al., 1998]. Akt, a serine/threonine kinase that mediates survival signals to protect against apoptosis [Anmed et al., 1997; Dudek et al., 1997], is degraded by a caspase-mediated cleavage process, which results in a decrease of its kinase activity and causes survival pathways to be turned off for the irreversible commitment to cell death [Rokudai et al., 2000]. Lamin B_1, a major portion of the nuclear envelope lamins, which is cleaved by caspase-6 [Srinivasula et al., 1996] and releases a p28 fragment, is considered to be responsible for chromatin condensation, nuclear envelope breakdown, and DNA fragmentation [Neamati et al., 1995; Rao et al., 1996]. DFF45/ICAD, is a caspase-3 substrate that must be cleaved before apoptotic internucleosomal DNA fragmenta-

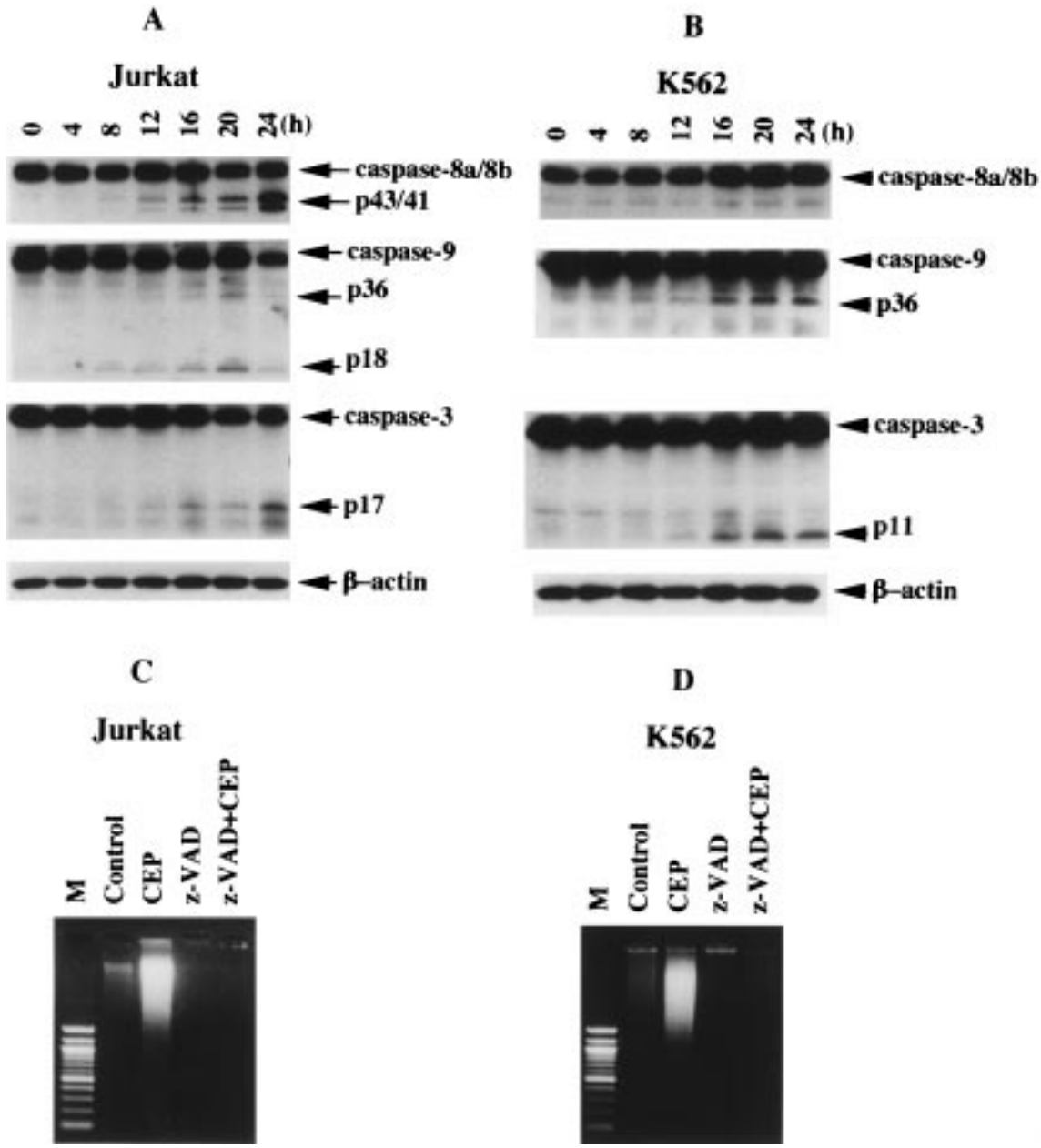

Fig. 5. Caspases are activated during CEP-induced apoptosis in Jurkat and K562 cells. Jurkat **(A)** and K562 **(B)** cells were incubated with 10 μM of CEP for the indicated times, and processing of procaspases was detected by immunoblotting analyses. The blotted membrane was consecutively stained with caspase-8, -9, -3, and β-actin antibodies after stripping the former staining. Positions of the intact and cleaved forms of caspases are indicated by arrows. In addition, Jurkat **(C)** and K562 **(D)** cells were pretreated with 100 μM of zVAD-fmk for 30 min and then incubated with 10 μM of CEP for 20 h. DNA fragmentation was analyzed by agarose gel electrophoresis.

tion proceeds [Liu et al., 1997; Enari et al., 1998]. DFF40/CAD remains inactive while bound to DFF45/ICAD. However, caspase-3 cleaves DFF45/ICAD, thereby releasing CAD, which then cleaves DNA to produce interchromosomal DNA fragmentation [Sakahira et al., 1998; Wolf et al., 1999]. In all of our cleavage experiments, Jurkat and K562 cells were treated with 10 μM of CEP for 4 to 24 h. As shown in Figure 6A and B, CEP induced cleavage of PARP, Bid, laminB_1, and DFF45/ICAD time-dependently in both Jurkat and K562 cells. The degrees of CEP-induced cleavage in K562 cells were generally lower than

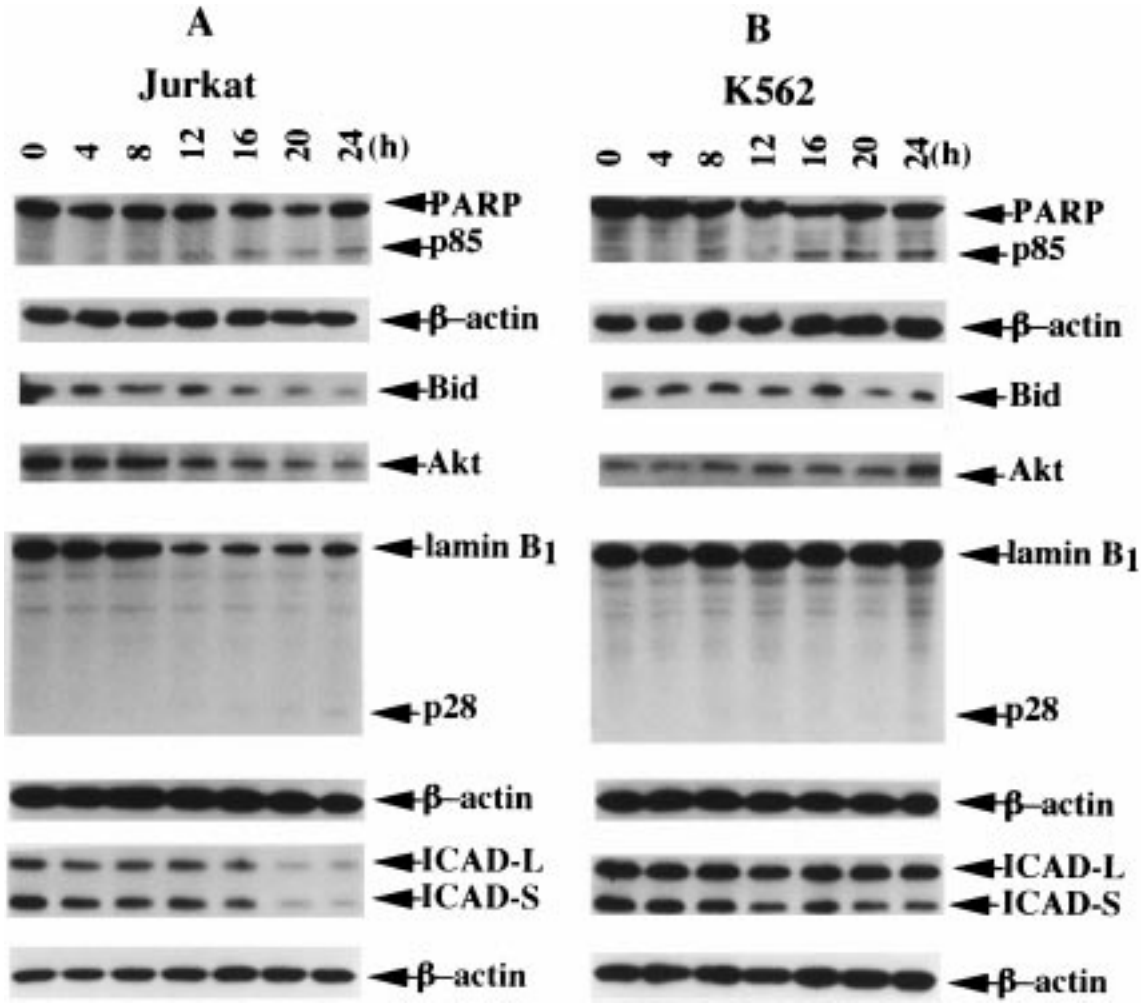

Fig. 6. Cleavage analyses of the caspase substrates, PARP, Bid, Akt, lamin B_1, and DFF45/ICAD, in CEP-treated Jurkat and K562 cells. Jurkat **(A)** and K562 **(B)** cells were incubated with 10 μM of CEP for the indicated times, and cleavage of cellular caspase substrates was analyzed by consecutively immunoblotting with PARP, Bid, Akt, lamin B_1, ICAD, and β-actin antibodies after stripping the former staining. Positions of the intact and processing forms of substrates are indicated by arrows.

those in Jurkat cells. For example, CEP triggered the cleavage of both ICAD-L and ICAD-S in Jurkat cells but only ICAD-S cleavage in K562 cells. More evidently, CEP induced the cleavage of Akt only in Jurkat cells. These results may partially explain the delayed and milder apoptosis in K562 cells after CEP treatment.

Degradation of P210 Bcr-Abl Oncoprotein During CEP-Induced Apoptosis in K562 Cells

Bcr-Abl is a chimeric oncoprotein that exhibits enhanced tyrosine kinase activity and is implicated in the pathogenesis of Philadelphia chromosome (Ph^1)-positive human leukemias [Konopka et al., 1984]. Bcr-Abl-mediated resistance to apoptosis was reported to be correlated with the up-regulation of Bcl-X_L [Amarante-Mendes et al., 1998a], and some cytotoxic agents have been shown to induce apoptosis in Bcr-Abl-positive cells by down-regulating Bcl-X [Oetzel et al., 2000]. Because CEP induced delayed but obvious apoptosis in K562 cells, we speculated that degradation of p210 Bcr-Abl and/or Bcl-X_L in K562 cells might have taken place during the process of apoptosis. To test this possibility, we measured the levels of Bcr-Abl protein by treating K562 cells with 10 μM of CEP for 4 to 24 h. As shown in Figure 7A, p210 Bcr-Abl protein was progressively degraded in a time-dependent manner upon CEP treatment. In contrast, Bcl-X_L was rather stable upon CEP treatment (Fig. 7B). To explore whether caspases are related to the degradation of Bcr-Abl,

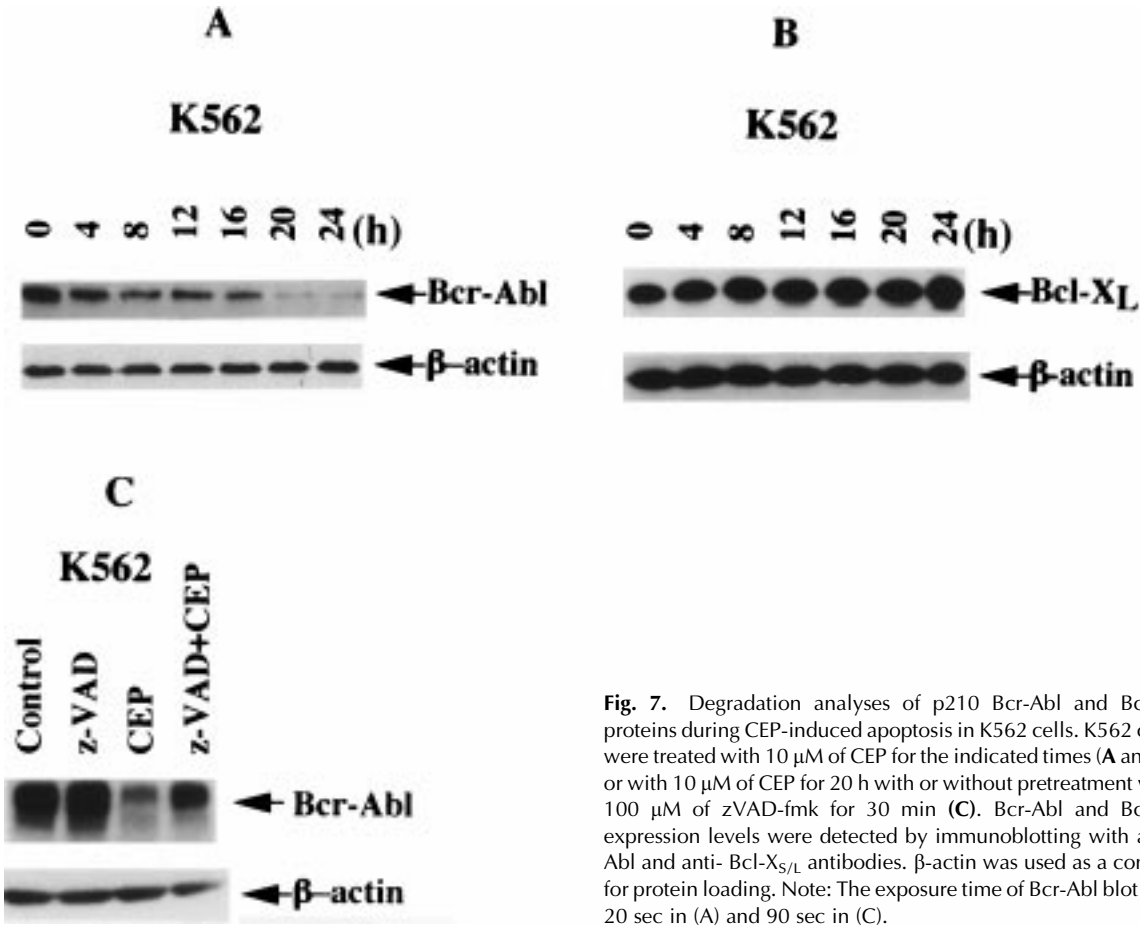

Fig. 7. Degradation analyses of p210 Bcr-Abl and Bcl-X_L proteins during CEP-induced apoptosis in K562 cells. K562 cells were treated with 10 μM of CEP for the indicated times (**A** and **B**) or with 10 μM of CEP for 20 h with or without pretreatment with 100 μM of zVAD-fmk for 30 min **(C)**. Bcr-Abl and Bcl-X_L expression levels were detected by immunoblotting with anti-Abl and anti- Bcl-$X_{S/L}$ antibodies. β-actin was used as a control for protein loading. Note: The exposure time of Bcr-Abl blot was 20 sec in (A) and 90 sec in (C).

the general caspase inhibitor zVAD-fmk (100 μM) was added 30 min before treatment with 10 μM of CEP (20 h). As shown in Figure 7C, zVAD-fmk partially blocked the degradation of Bcr-Abl.

Herbimycin A Enhances CEP-Induced Apoptosis in K562 Cells

Herbimycin A (HMA), a benzoquinonoid ansamycin antibiotic, is a selective protein tyrosine kinase (PTK) inhibitor [Uehara et al., 1989]. Treatment of cells with HMA was previously shown to cause irreversible degradation of p210 Bcr-Abl protein, thereby resulting in decreased tyrosine kinase activity of p210 Bcr-Abl [Anafi et al., 1993]. Recent evidence has also demonstrated that HMA accelerates anticancer drug- and γ-irradiation-induced apoptosis in Bcr-Abl-positive leukemia cells [Riordan et al., 1998]. Accordingly, we assumed that HMA might sensitize K562 cells to CEP treatment for the induction of more extensive apoptosis. To test this assumption, we pretreated K562 cells with a noncytotoxic concentration of HMA (400 ng/ml) [Anafi et al., 1993], 1 h before the 10 μM of CEP stimulation (20 h). As shown in Figure 8A, pretreatment with this concentration of HMA markedly promoted apoptosis induced by 2, 5, or 10 μM of CEP as determined by flow cytometric analysis. Consistent results were also obtained by DNA fragmentation analysis in agarose gel electrophoresis (Fig. 8B). In contrast, HMA did not influence CEP-induced apoptosis in Jurkat cells (data not shown). In order to study the molecular mechanism of the enhanced sensitivity to CEP in HMA-treated K562 cells, we investigated the change in protein levels of Bcr-Abl, Akt, and Bcl-X_L during this process. As shown in Figure 8C and D, remarkable degradation or reduction in the amounts of p210 Bcr-Abl and Akt proteins in HMA-treated and HMA + CEP-treated K562 cells was observed, and this might have contributed to the enhancement of CEP-

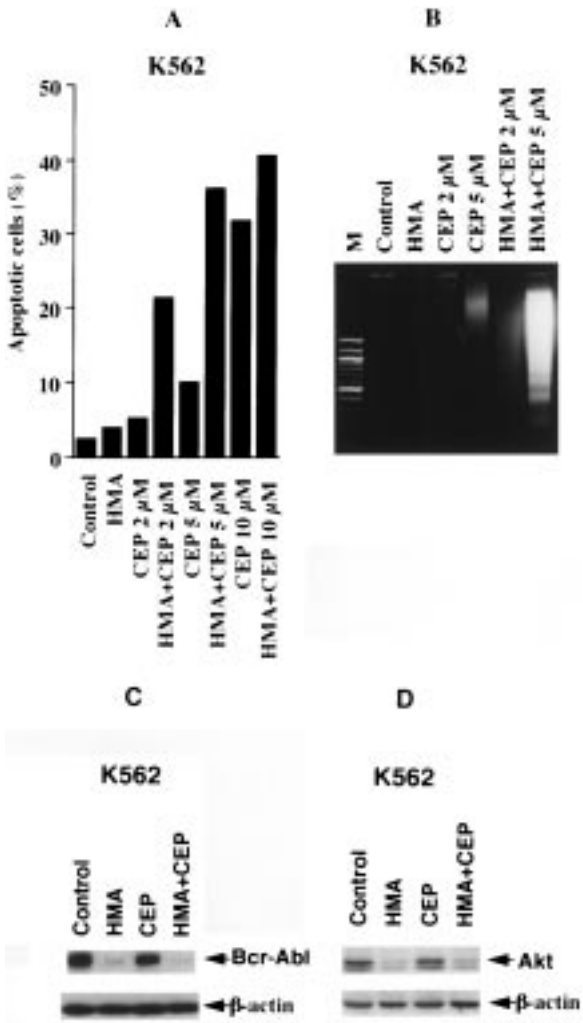

Fig. 8. Herbimycin A sensitizes Bcr-Abl-positive K562 cells for CEP-induced more extensive apoptosis. K562 cells were pretreated with 400 ng/ml HMA for 1 h and then incubated with the indicated concentrations of CEP for 20 h. They were then subjected to flow cytometry for quantitative measurement of apoptotic cells **(A)** or to analysis for DNA fragmentation by agarose gel electrophoresis **(B)**. In addition, HMA-pretreated K562 cells were incubated with 10 μM of CEP for 12 h and analyzed for Bcr-Abl **(C)** and Akt **(D)** protein levels by immunoblotting with anti-Abl or anti-Akt antibody. β-actin was used as a control for protein loading.

induced apoptosis. However, we did not find changes in Bcl-X_L expression levels during this process (data not shown).

DISCUSSION

In this study, we found that CEP induces time- and dose-dependent apoptosis in human leukemia Jurkat T cells and K562 CML cells. DNA fragmentation was induced in Jurkat cells by low concentrations of CEP (1–5 μM). CEP also induced DNA fragmentation in K562 cells, although it was necessary to incubate with higher concentrations of CEP and for longer times compared with Jurkat cells. In addition, we found that CEP treatment induced typical apoptotic morphological changes such as condensed chromatin and fragmented nuclei in both of the cell lines.

Recent findings have suggested that both caspase and mitochondria play key and central roles in apoptosis regardless of the stimuli [Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997; Green and Reed, 1998]. In the present study, we investigated and partially defined the pathway of apoptotic signal transduction of CEP-induced apoptosis in Jurkat and K562 cell lines. In both types of cells, we found that CEP induced disruption of the mitochondria $\Delta\psi_m$ at the early stage of apoptosis. It is known that Bcl-2, Bcl-X_L, and Bax, Bcl-2 family members, play important roles in the mitochondrial apoptosis pathway. K562 cells do not express significant levels of Bcl-2 [Dubrez et al., 1998], and we did not find any down-regulation of Bcl-2 in Jurkat cells (data not shown) or Bcl-X_L down-regulation in K562 cells (Fig. 7B) or up-regulation of Bax in both types of cells (data not shown) when the cells were treated with 10 μM of CEP for 4 to 24 h. Therefore, CEP, which has a hydrophobic structure and can readily permeate into cells [Shiraishi et al., 1987], might directly target mitochondria to cause damage to the michondrial outer membrane for disruption of mitochondrial $\Delta\psi_m$, not through changing Bcl-2, Bcl-X_L or Bax levels. The damaged mitochondria might release cyt. c, which activates caspase-9 and downstream caspase-3 but not caspase-8 [Fulda et al., 1998]. In Jurkat cells, we found that CEP treatment induced activations of caspase-8, in addition to caspase-9 and -3. On the other hand, we found that this caspase-8 activation was Fas-independent (Fig. 4). It was previously reported that AIF, which was purified from the mitochondria, could activate both caspase-8 and -3 [Susin et al., 1997; Fulda et al., 1998]. However, the same research group has recently demonstrated that molecularly cloned AIF induces an entirely caspase-independent process of apoptosis [Susin et al., 1999; Daugas et al., 2000]. Thus, CEP-induced caspase-8 activation in Jurkat cells may be triggered by a yet-unknown mechanism. Additionally, our results showed that Bid was cleaved during

CEP-induced apoptosis in Jurkat cells (Fig. 6A), probably by the activated caspase-8 and -3 [Bossy-Wetzel and Green, 1999]. While full-length Bid is localized in the cytosol, truncated Bid (tBid) translocates to mitochondria and thus transduces apoptotic signals to mitochondria [Li et al., 1998; Luo et al., 1998]. Thus, such an apoptotic self-amplifying feedback loop may operate in CEP-treated Jurkat cells. We lastly found that the caspases activated by CEP actually cleaved a number of substrates such as Akt, lamin B_1, PARP, and DFF45/ICAD, which ultimately led to cleavage of DNA for the fragmentation in Jurkat cells.

Our results also showed that treatment of K562 cells with CEP led to disruption of the mitochondria $\Delta\psi_m$, activations of caspase-9 and -3, and cleavage of Bid, lamin B_1, PARP, and DFF45/ICAD. However, caspase-8 was not activated in these cells during the CEP-triggered apoptotic process. Moreover, caspase-mediated cleavage of a number of substrates in K562 cells was generally less evident than that in Jurkat cells, and Akt was totally resistant to CEP-induced cleavage in K562 cells, though caspase-3 was activated. This might make K562 cells reserve some survival signals derived from Akt kinase, which could be one reason for their reduced sensitivity to CEP treatment.

The p210 form of Bcr-Abl is seen in 95% of patients with CML and up to 20% of adult patients with de novo acute lymphocytic leukemia [Shtivelman et al., 1985; Clark et al., 1988]. K562 cells, which express p210 Bcr-Abl, have been shown to be highly resistant to antileukemic drug- and γ-irradiation-induced apoptosis [McGahon et al., 1994; Nishii et al., 1996]. It has been reported that Bcr-Abl delays apoptosis by blocking cyt. c release and caspase-3 activation [Dubrez et al., 1998; Amarante-Mendes et al., 1998b]. Because CEP rather exceptionally induced apoptosis in Bcr-Abl-bearing K562 cells, we examined whether antiapoptotic Bcr-Abl is degraded during CEP-induced apoptosis. Actually, our results showed that the expression level of Bcr-Abl in CEP-treated K562 cells was remarkably down-regulated in a time-dependent manner, and this degradation of Bcr-Abl was prevented to some extent by zVAD-fmk pretreatment. These findings suggest that caspases play a role in the degradation of antiapoptotic Bcr-Abl protein, although other mechanisms may also be involved in this process. In any case, our results suggest that the caspase-related degradation of antiapoptotic p210 Bcr-Abl plays an important role in the acceleration of CEP-induced apoptotic cell death in K562 cells.

The selective tyrosine kinase inhibitor HMA was previously identified as an effective differentiating agent of K562 cells [Honma et al., 1989]. In order to overcome the resistance to CEP-induced programmed cell death signal in K562 cells, we investigated whether nanogram levels of HMA are capable of enhancing the induction of apoptosis by CEP. We found that HMA did increase the sensitivity of K562 cells to CEP-induced apoptosis. This was confirmed by both agarose gel electrophoretic and flow cytometric analyses of apoptosis. The mechanism of synergistic activity by a nontoxic concentration of HMA is intriguing. Anafi et al. [1993] and we (Fig. 8C) verified the almost complete degradation of the p210 Bcr-Abl protein in the presence of a nonapoptotic concentration of HMA. We also found that 400 ng/ml of HMA triggered obvious G_1 phase arrest of K562 cells (about 81%) after 21 h of incubation (data not shown), although it did not induce apoptosis (Fig. 8A and B). We have also demonstrated that a nontoxic concentration of HMA induces a remarkable decrease in the Akt protein level, which is otherwise stable in CEP-treated K562 cells. Further research is needed to clarify whether the reduction in the amount of Akt proteins due to HMA is related to Bcr-Abl degradation. More recently, it has been reported that HMA can inhibit the chaperone association of Bcr-Abl with Hsp-90 and promote the degradation of Bcr-Abl through the proteasomes [An et al., 2000]. We speculate that HMA-induced G_1 phase arrest and degradation of the antiapoptotic proteins Bcr-Abl and Akt may enhance the apoptosis-inducing signals caused by CEP and lead to more extensive apoptosis in K562 cells, although the inhibitory effect of HMA on other protein kinases providing survival signals may also be involved in the process.

A pharmacokinetic study of CEP has shown that a plasma concentration of about 1–2 μg/ml (1.35–2.70 μM) did not cause any adverse effects in volunteer subjects [Yasuda et al., 1989]. This suggests that low concentrations of CEP, which were shown to induce apoptosis in our study, may be clinically achievable.

In summary, our results indicate that CEP induces apoptosis of human leukemia Jurkat T cells and CML K562 cells in a caspase-depen-

dent and Fas-independent manner. We have demonstrated that the p210 Bcr-Abl oncoprotein is time-dependently degraded during CEP-induced apoptosis in K562 cells. Our experimental study using human leukemic cell lines indicates that CEP may be useful for the treatment of some types of leukemia and that combinations of CEP and HMA might exert some therapeutic effects on CML.

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