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Induction of Apoptosis by Bufalin in Human Tumor Cells Is Associated with a Change of Intracellular Concentration of Na^+ Ions[1]

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In an attempt to characterize the mechanisms that are operative at the early stages of the induction of apoptosis by bufalin, a component of the traditional Chinese medicine *chan'su*, we examined the effects of bufalin on plasma membrane potential, as determined by monitoring the uptake by cells of rhodamine 123. Bufalin induced apoptosis in human monocytic leukemia THP-1 cells, in human lymphoblastic leukemia MOLT-3 cells, and in human colon adenocarcinoma COLO320DM cells but not in normal human leukocytes, for example, polymorphonuclear cells and lymphocytes, and not in murine leukemia P388D1 and M1 cells. Treatment for 3 h with bufalin at 10^{-6} M caused a decrease in the plasma membrane potential in several lines of human tumor cells but not in murine leukemia cells. No changes in mitochondrial membrane potential, as monitored with the fluorescent dye JC-1, and no release of cytochrome *c* were observed within at least 6 h after the start of treatment with bufalin. Moreover, overexpression of *bcl-2* in human leukemia HL60 cells that had been transfected with cDNA for *bcl-2* prevented bufalin-induced apoptosis but had no significant effect on the change in plasma membrane potential induced by bufalin. Since bufalin specifically inhibits the Na^+,K^+-ATPase of human but not murine tumor cells, and since this inhibition leads to a change in intracellular concentration of Na^+ ions, our findings suggest that bufalin induces apoptosis in human tumor cells selectively *via* inhibition of the Na^+,K^+-ATPase, which acts upstream of the bcl-2 protein.

Key words: apoptosis, bufalin, tumor cell, membrane potential, bcl-2.

Apoptosis is a genetic process that leads to cell death. It is distinct from necrosis (*1*) and is thought to play important roles in embryogenesis (*2*), carcinogenesis (*3*), regulation of the maturation of the immune system (*4, 5*), and the cytotoxic cell killing of virally infected cells (*6*). Apoptosis was originally defined in terms of characteristic changes in morphology (*7*), which include cell shrinkage, the condensation of chromatin in the nucleus, and the digestion of chromatin into DNA fragments with lengths that are multiples of about 180 bp. At the same time, the plasma membrane and cytoplasmic organelles remain relatively intact. Mechanisms of induction of apoptosis have been studied extensively. Overexpression of the *bcl-2* gene, which was originally identified at the t(14;18) (q32;q21) breakpoint in human lymphoid malignancies (*8, 9*), in lymphoid cells in culture (*4, 10*) and in transgenic mice (*11, 12*) prevents apoptosis that otherwise occurs in response to a wide variety of agents.

We reported previously that bufalin, a major component of the traditional Chinese medicine *chan'su*, which is prepared from toad venom, induces apoptosis in human leukemia HL60 cells (*13*) and U937 cells (*14*). The first target of bufalin is thought to be the Na^+,K^+-ATPase, since bufalin and the structurally related bufadienolides have been shown to inhibit this enzyme (*15*). In the present study, we investigated the induction of apoptosis by bufalin in normal human leukocytes and several lines of human and murine tumor cells other than HL60 and U937, and we examined the involvement of a change in intracellular concentration of Na^+ ions resulting from the inhibition of Na^+,K^+-ATPase at the early stages of bufalin-induced apoptosis.

MATERIALS AND METHODS

Materials—Bufalin, RNase A, 4′,6-diamidino-2-phenylindole (DAPI) and rhodamine 123 were obtained from Sigma Chemical (St. Louis, MO). G418 (geneticin disulfate) was purchased from Wako Pure Chemical Industries (Osaka). JC-1 was obtained from Molecular Probes (Eugene, OR).

Cells and Cell Culture—Tumor cells (THP-1, K562, MOLT-3, HL60, U937, P388D1, and COLO320DM) were provided by the Japanese Cancer Research Resources Bank (Tokyo). All cells used in this study were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum at 37°C in humidified atmosphere of 5% CO_2 in air.

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Cultures of HL60 cells that had been transfected with the control vector or with this vector plus *bcl-2* cDNA were grown as described above in the presence of 500 μg/ml G418. Na^+-deficient medium was prepared by replacing all sodium salts in RPMI-1640 with potassium salts.

Separation of Peripheral Blood Cells—Normal human leukocytes (monocytes, polymorphonuclear cells, and lymphocytes) were prepared from heparinized human blood that had been obtained from a healthy donor as described previously (*16*). Peripheral blood was diluted with an equal volume of PBS(−) and layered on a cushion of Histopaque-1077 (Sigma Chemical). After centrifugation at 250 × *g* for 30 min at room temperature, the cells at the plasma-medium interface, which included the mononuclear cells, and the medium below the interface, which contained polymorphonuclear cells (PMN), were separately collected. The mononuclear cells were washed free of platelets by centrifugation at 40 × *g* for 5 min and allowed to attach to culture plates for 1 h at 37°C. The adherent cells were regarded as monocytes, and the nonadherent cells were considered to be lymphocytes. The purity of the final preparation of mononuclear cells, as determined by peroxidase staining, was greater than 85%. The PMN cells were purified from the interface after centrifugation at 250 × *g* for 30 min at room temperature on Mono-poly Resolving Medium (Flow Laboratories, Irvine, Scotland). The purity of PMN cells was greater than 95%, as judged by morphological examination after May-Gruenwald-Giemsa staining.

Morphological Analysis of Apoptotic Cells—Cells were fixed, subjected to May-Gruenwald-Giemsa staining, and processed for oil-immersion microscopy. Apoptotic cells were identified as cells with condensed and fragmented nuclei, and these cells were counted.

Detection of DNA Fragmentation—DNA was prepared for gel electrophoresis as described previously (*17*). Electrophoresis was performed in 1% agarose gels in 40 mM Tris-acetate buffer (pH 7.4) at 50 V for 1 h. The fragmented DNA was visualized by staining with ethidium bromide after electrophoresis.

Quantitation of DNA Fragmentation—The extent of DNA fragmentation was determined as previously described (*18, 19*). In brief, cells were harvested by centrifugation and lysed by incubation in 0.5% Triton X-100 that contained 5 mM Tris-HCl (pH 7.4) and 1 mM EDTA for 20 min on ice. The lysate and the supernatant obtained after centrifugation at 27,000 × *g* for 20 min were sonicated for 40 s, and the DNA content was measured with the fluorescent reagent DAPI by a fluorometric method. The percentage of fragmented DNA was defined as the ratio of the DNA content of the supernatant to that of the lysate multiplied by 100.

Measurement of Membrane Potential—The flow cytometric assay that we used provides an estimate of membrane potential on the basis of the partitioning of a lipophilic cationic fluorescent dye, rhodamine 123, between cells and the medium in which they are suspended. Rhodamine 123 is a fluorescent dye that is highly specific for mitochondria in living cells. The plasma membrane potential of cells can be monitored in media, in which the mitochondrial membrane potential remains constant (*20*). The plasma membrane potential of cells is dependent on the extracellular and intracellular concentrations of K^+ and Na^+ ions. Thus, inhibition of the Na^+,K^+-ATPase induces a decrease in membrane potential (depolarization) reflected in the uptake of rhodamine 123 by the cells. Cells were treated with bufalin and resuspended in PBS(−) that contained 5 mM glucose. Rhodamine 123 (2 μM) was added to the suspension, which was then incubated for 30 min at 37°C. The green (520–530 nm) fluorescence of rhodamine 123 in individual cells was determined with a flow cytometer (EPICS Elite; Coulter, Hialeah, FL). For excitation of the probe, light was provided at 488 nm by an ionic argon laser. The mean cellular fluorescence of rhodamine 123 was recorded.

Preparation of an S-100 Fraction and Immunoblotting Analysis of the Release of Cytochrome c from Mitochondria—Cells were washed twice with ice-cold PBS(−) and resuspended in five volumes of buffer A (20 mM HEPES-KOH, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF, and 250 mM sucrose). The cells were homogenized by passage through a 22-gauge needle, and the homogenate was centrifuged at 100,000 × *g* for 15 min at 4°C. The supernatant (S-100 fraction) was collected and subjected to immunoblotting with antibodies for cytochrome *c*. After electrophoresis on SDS-polyacrylamide gel (15% w/v polyacrylamide), bands of protein were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and immunoblotted with purified mouse monoclonal antibodies against cytochrome *c* (Pharmigen, San Diego, CA).

Construction of an Expression Vector and Transfection of Cells—The 0.9-kb *Eco*RI fragment that contained the open reading frame of the cDNA for human *bcl-2* was inserted into the *Hin*cII site of the pUC19 cloning vector. The insert was cut with *Xba*I and *Hin*dIII and subcloned into the *Xba*I and *Hin*dIII restriction sites of the pRc-RSV expression vector. Transfections were performed by electroporation with a Gene Pulsor (Bio-Rad, Hercules, CA), as described previously (*21*). The pulse was delivered to 0.4 ml of a suspension of HL60 cells (2×10^6 cells) that contained 10 μg of plasmid DNA. Then cells (2×10^4 cells per well) were plated in 96-well microplates and cultured in RPMI-1640 medium. After culture for 72 h, G418 was added at a final concentration of 1 mg/ml. Subclones were isolated four to 6 weeks later.

Northern Blotting Analysis—Total RNA was prepared as described by Chomczynski and Sacchi (*22*), and aliquots of 15 μg of RNA were subjected to electrophoresis on 1% denaturing agarose gels that contained formaldehyde. Bands of RNA were transferred onto a Hybond-N membrane (Amersham, Buckinghamshire, UK). Northern blotting analysis was performed as described previously (*23*).

RESULTS

Induction of Apoptosis in Human but Not Murine Tumor Cells by Bufalin—We showed previously that bufalin induces apoptosis in human promyelocytic leukemia HL60 cells (*13*). In the present study, we examined the effects of bufalin on various lines of tumor cells and normal leukocytes. Figure 1 shows the morphological changes in tumor cells that had been treated with 10^{-5} M bufalin for 18 h. Human colon adenocarcinoma COLO320DM cells and acute monocytic leukemia THP-1 cells developed obvious apoptotic features that included chromatin condensation and

nuclear fragmentation (Fig. 1, B and D). By contrast, almost no morphological changes were evident in murine leukemia P388D1 cells (Fig. 1F) and M1 cells (data not shown). These results suggest that bufalin might selectively induce apoptosis in tumor cells of human origin but not in those of murine origin.

The extent of DNA fragmentation induced by bufalin was quantified by a method that involved staining with DAPI (Fig. 2). Significant increases in DNA fragmentation were detected in human tumor cells, such as COLO320DM, THP-1, and lymphoblastic leukemia MOLT-3 cells, after the treatment with bufalin at concentrations above 10^{-8} M for 24 h (Fig. 2). However, barely any fragmentation of DNA was detected in murine P388D1 cells that had been treated with bufalin, even when the concentration of the drug was as high as 10^{-5} M. Under the same experimental conditions, bufalin also induced DNA fragmentation in other lines of human leukemia cells, such as K562 and KG-1 cells (data not shown).

Figure 3 shows the results of agarose gel electrophoresis of DNA extracted from monocytes and lymphocytes, namely, normal cells, and from acute monocytic leukemia THP-1 cells and acute lymphoblastic leukemia MOLT-3 cells that had been treated with bufalin for various times. A ladder pattern typical of internucleosomal fragmentation developed in the case of leukemia THP-1 cells. The ladder pattern in the case of MOLT-3 cells was less clear (Fig. 3C), but the bufalin-induced fragmentation of DNA was con-

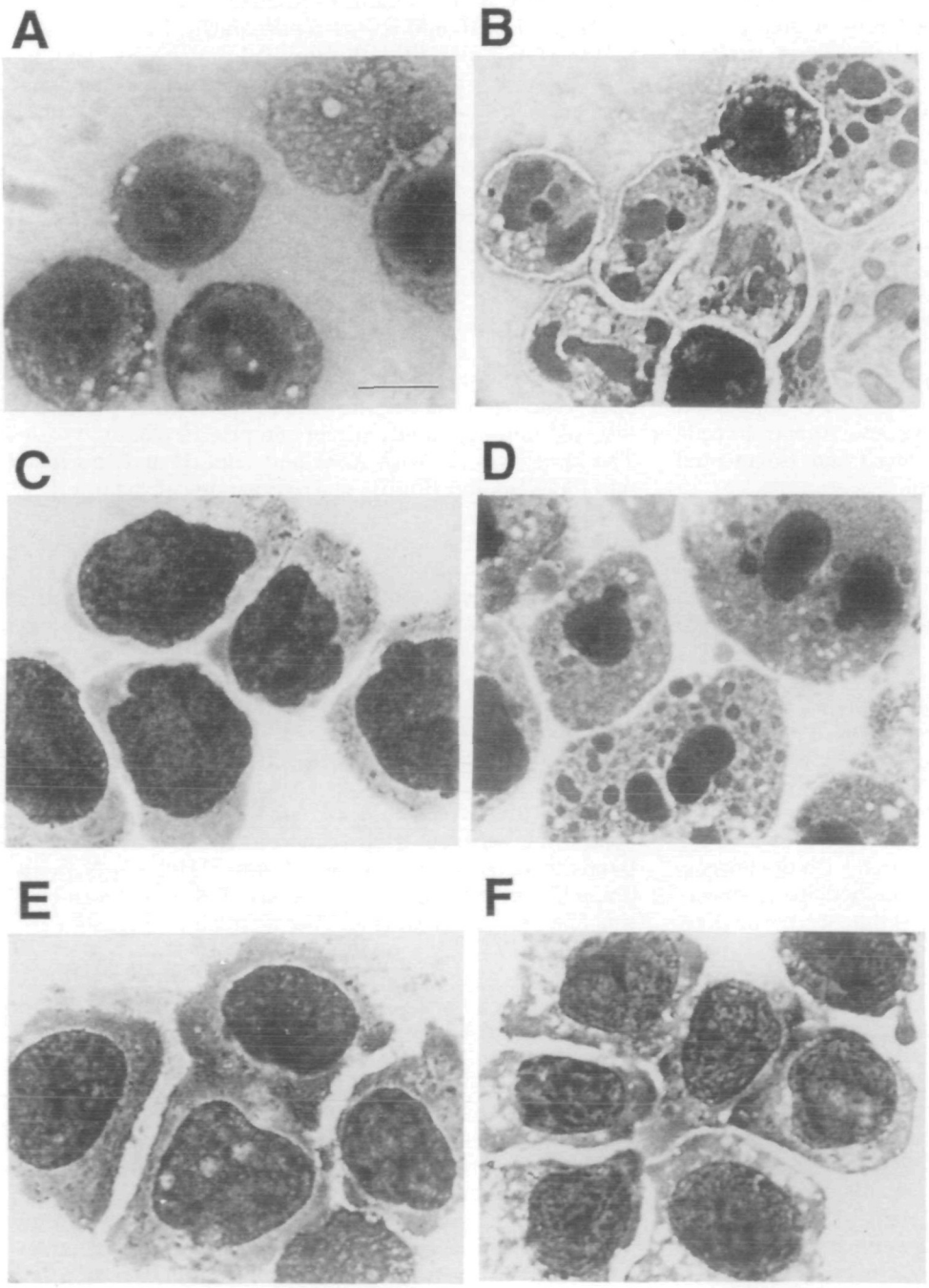

Fig. 1. **Morphological appearance of human and murine tumor cells after treatment with bufalin.** Human colon tumor COLO320-DM cells (A, B), human monocytic leukemia THP-1 cells (C, D), and murine leukemia P388D1 cells (E, F) were treated with 10^{-5} M bufalin for 18 h and subjected to May-Giemsa staining. A, C, and E, Untreated cells; B, D, and F, cells treated with bufalin. The bar indicates 10 μm.

firmed by staining fragmented DNA with DAPI (data not shown). By contrast, no DNA fragmentation was induced in human normal leukocytes that had been treated with 10^{-6} M bufalin (Fig. 3, B and D). Our findings suggest that bufalin can induce apoptosis in various lines of human tumor cells but not in murine leukemia cells or in normal human leukocytes.

Effects of Bufalin on Membrane Potential—Bufalin inhibits the activity of Na^+,K^+-ATPase (*24*), the enzyme that catalyzes the coupled active transport of Na^+ and K^+ ions across the plasma membrane of most animal cells and maintains the membrane potential. We examined the effects of treatment with bufalin at 10^{-6} M on the membrane potential of various tumor cells by monitoring the fluorescence of rhodamine 123 that was incorporated into cells. Figure 4A shows the results of flow-cytometric analysis of changes in membrane potential of THP-1 cells that had been treated with 10^{-6} M bufalin for 3 and 6 h. The membrane potential of cells was expressed in terms of the intensity of fluorescence of rhodamine 123 that had been incorporated into cells in the largest individual population of cells. The number of THP-1 cells with a lower than normal membrane potential increased with the duration of treatment with bufalin. By contrast, the membrane potential of murine P388D1 cells was unchange during prolonged treatment with 10^{-6} M bufalin (Fig. 4B). Figure 4C shows the changes in membrane potential of various tumor cells that had been treated with 10^{-6} M bufalin for the indicated times and then analyzed by flow cytometry. The membrane potentials of human leukemia THP-1, HL60, and U937 cells, as well as that of human colon adenocarcinoma COLO320DM cells, were markedly reduced by the treatment with bufalin, whereas that of murine P388D1 cells was unaffected by bufalin. These findings suggest that the Na^+,K^+-ATPase of human tumor cells might be inhibited by bufalin, while that of murine tumor cells is not.

Inhibition of Na^+,K^+-ATPase by bufalin might result in a change of intracellular Na^+ concentration. To determine whether or not the change of intracellular Na^+ concentration resulting from the inhibition of Na^+,K^+-ATPase by bufalin may have induced apoptosis in HL60 cells, the cells were treated with bufalin in Na^+-deficient RPMI-1640 medium prepared as described in "MATERIALS AND METHODS." As shown in Fig. 5, treatment of HL60 cells with 10^{-6} M bufalin for 6 and 8 h caused successive increase in DNA fragmentation. When HL60 cells were incubated in Na^+-deficient medium for 6 and 8 h, a slight increase of DNA fragmentation was observed as compared to that detected in normal RPMI-1640 medium. However, no significant effect of bufalin on the induction of DNA fragmentation was observed in HL60 cells by treatment with 10^{-6} M bufalin for 6 and 8 h in the Na^+-deficient medium (Fig. 5). Similar results were obtained with U937 cells upon treatment with bufalin in Na^+-deficient medium (data not shown). These results suggest that the change of intracellular Na^+ concentration resulting from the inhibition of Na^+,K^+-ATPase by bufalin might play an important role in the induction of apoptosis by bufalin.

Effects of Bufalin on the Release of Cytochrome c and Plasma Membrane Potential Change—Since the uptake of the fluorescent dye rhodamine 123 is affected both by the plasma membrane potential and by the mitochondrial membrane potential, we examined whether the decrease in the amount of rhodamine 123 incorporated into cells might reflect a change in the plasma membrane potential and not in the mitochondrial membrane potential. We examined changes in the mitochondrial membrane potential by

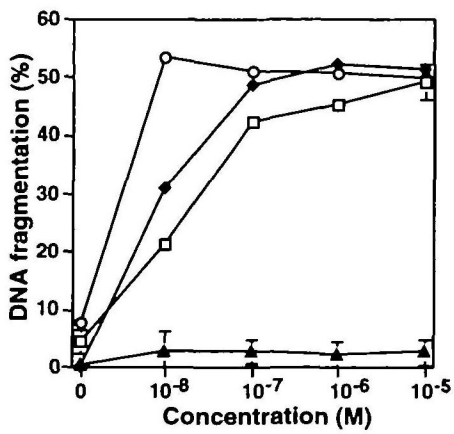

Fig. 2. **The kinetics of DNA fragmentation induced by treatment with bufalin.** The extent (%) of DNA fragmentation was determined by staining DNA with DAPI as described in "MATERIALS AND METHODS." MOLT-3 (○), THP-1 (◆), COLO320DM (□), and P388D1 (▲) cells were treated with bufalin at various concentrations for 24 h. Data presented are the means ± SD of results from three independent experiments.

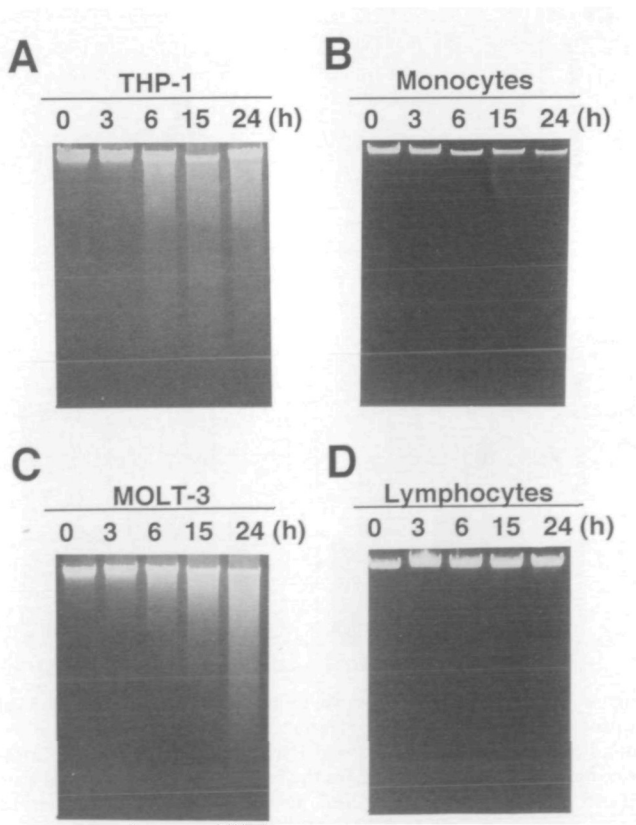

Fig. 3. **Agarose gel electrophoresis of DNA extracted from human leukemia cells and normal leukocytes after treatment with 10^{-6} M bufalin for the indicated times.** Cellular DNA was extracted and analyzed as described in "MATERIALS AND METHODS." A, monocytic leukemia THP-1 cells; B, monocytes; C, lymphoblastic leukemia MOLT-3 cells; and D, lymphocytes.

measuring the incorporation of the fluorescent dye JC-1, which has been reported to reflect changes in mitochondrial membrane potential (*24*), but we observed no significant changes within 6 h after the start of treatment with bufalin (data not shown). Moreover, as shown in Fig. 6A, the release of cytochrome *c* from mitochondria, which is known to occur upon a change in mitochondrial membrane potential, started 9 h after the start of treatment with bufalin, continued for 15 h, and disappeared at 24 h. These results suggest that bufalin had no effect on the mitochondrial membrane potential within at least the first 6 h after the start of treatment with bufalin. Therefore, the decrease in membrane potential that we detected by monitoring the incorporation of rhodamine 123 into cells and that occurred within 6 h might reflect a change in the plasma membrane potential exclusively but not in the mitochondrial mem-

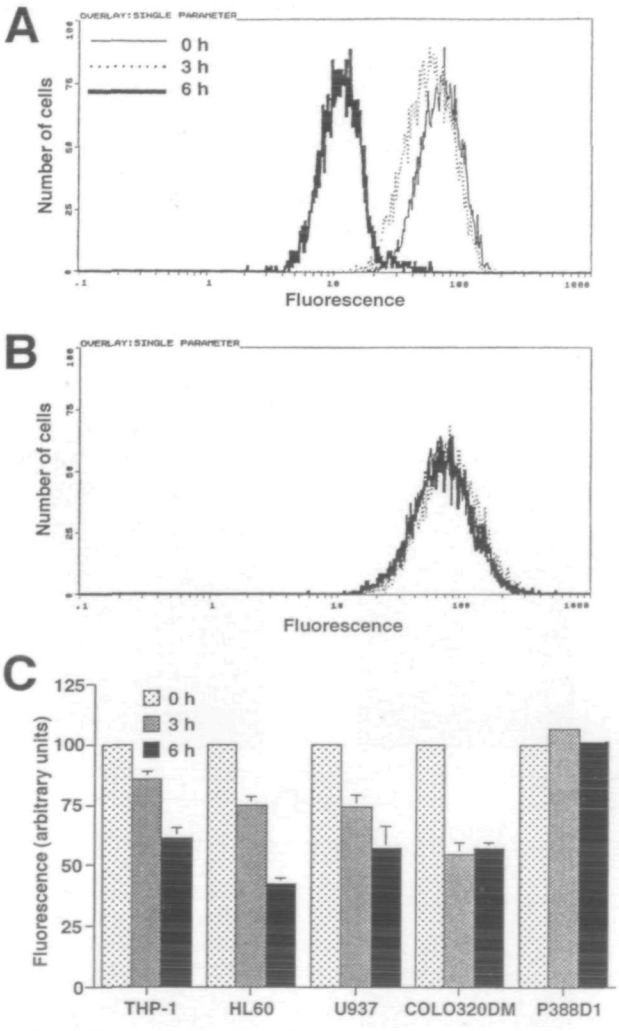

Fig. 4. **Effects of bufalin on the membrane potential of various tumor cells.** A and B: Flow-cytometric analysis of changes in the membrane potential of THP-1 and P388D1 cells, respectively, after treatment with 10^{-6} M bufalin for the indicated times. The cells were loaded with rhodamine 123 and analyzed by flow cytometry as described in "MATERIALS AND METHODS." ·, 0 h; ···, 3 h; –, 6 h. Panel C shows the changes in membrane potential of various tumor cells that had been treated with 10^{-6} M bufalin for the indicated times. The membrane potential was expressed as the intensity of fluorescence of rhodamine 123 incorporated into cells that comprised the largest population in each sample. Data presented are the means ± SD of results from three independent experiments.

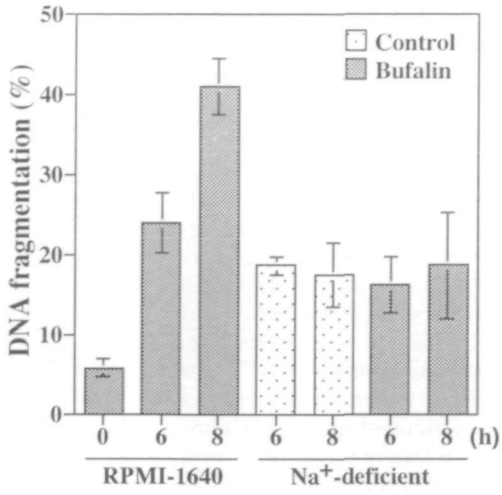

Fig. 5. **Inhibition of bufalin-induced DNA fragmentation in Na^+-deficient medium.** HL60 cells were treated with 10^{-6} M bufalin for the indicated times in normal RPMI-1640 medium or in Na^+-deficient medium. The extent (%) of DNA fragmentation was quantified by fluorometry with DAPI as described in "MATERIALS AND METHODS." Data are means ± SD of results from three independent experiments.

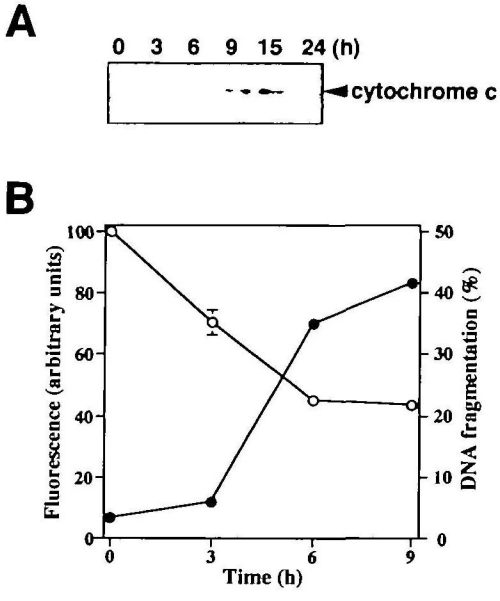

Fig. 6. **(A) Effects of bufalin on the release of cytochrome *c* from mitochondria.** Cytosolic levels of cytochrome *c* in THP-1 cells that had been treated with 10^{-6} M bufalin for the indicated times were measured by immunoblotting with antibodies against cytochrome *c*. **(B) Comparison of the time course of changes in the membrane potential of THP-1 cells in response to bufalin with the time course of DNA fragmentation.** THP-1 cells were treated with 10^{-6} M bufalin for the indicated times. The membrane potential (○) was analyzed by flow cytometry using rhodamine 123, and the extent (%) of DNA fragmentation (●) was determined using DAPI, as described in the legend to Fig. 2. Data are means ± SD of results from duplicate assays. Absence of a bar indicates that the SD lies within the symbol.

brane potential.

Figure 6B shows the time course of DNA fragmentation induced in THP-1 cells by 10^{-6} M bufalin together with the time course of changes in plasma membrane potential. The extent of DNA fragmentation was unchanged for 3 h but then increased sharply. By contrast, a significant change in membrane potential was apparent 3 h after the start of treatment with bufalin, and the membrane potential of treated THP-1 cells had fallen by approximately 50% within 6 h, as estimated by monitoring the uptake of rhodamine 123 (Fig. 6B). These results suggest that the change in plasma membrane potential in THP-1 cells induced by bufalin preceded the release of cytochrome *c* as well as fragmentation of DNA.

Effects of the Overexpression of bcl-2 on Apoptosis—We examined whether overexpression of the *bcl-2* gene in HL60 cells could inhibit the induction of apoptosis by bufalin. First, we evaluated the expression of *bcl-2* mRNA in several clones of G418-resistant cells by Northern blotting analysis (Fig. 7A). Although the expression of endogenous *bcl-2* mRNA was scarcely detectable in both the parental cells (HL60/$_{WT}$) and in cells transfected with the pRc-RSV vector alone (HL60/neo), a significant level of *bcl-2* mRNA was detected in two clones that had been transfected with a pRc-RSV vector that included the cDNA for human *bcl-2* (HL60/bcl-2-I and HL60/bcl-2-II). As shown in Fig. 7B, ladders of DNA fragments were clearly observed in the case of HL60/neo cells, which had been transfected with control vector, after treatment with bufalin at concentrations above 6×10^{-9} M for 24 h, while no DNA frgmentation was observed in HL60/bcl-2-II cells at concentrations of bufalin below 9×10^{-9} M. The extent of DNA fragmentation in HL60/neo and HL60/bcl-2 cells was quantified by staining with DAPI and is shown in Fig. 7C. In agreement with the results shown in Fig. 7B, bufalin acted to induce DNA fragmentation in HL60/neo cells in a dose-dependent manner. By contrast, DNA fragmentation in response to bufalin was markedly inhibited in both selected clones of HL60/bcl-2 cells. These results indicate that overexpression of the *bcl-2* gene suppressed the

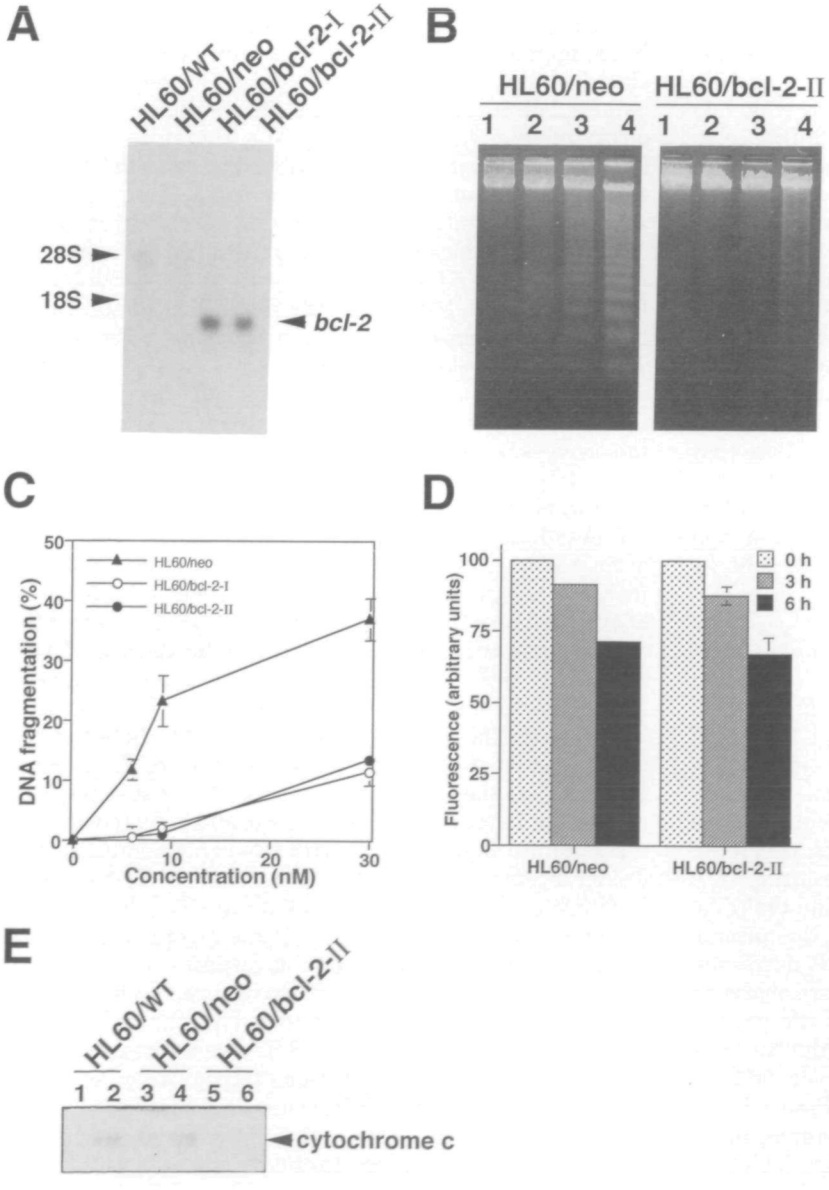

Fig. 7. **Effects of the overexpression of *bcl-2* on DNA fragmentation, the changes in plasma membrane potential, and release of cytochrome *c* induced by bufalin.** (A) Total RNA was prepared from the indicated cells. Aliquots of 15 μg of RNA were analyzed by Northern blotting. *bcl-2* mRNA was identified by hybridization with ^{32}P-labeled human *bcl-2* cDNA. HL60/$_{WT}$, wild-type HL60 cells; HL60/neo, cells transfected with the vector; HL60/bcl-2-I and HL60/bcl-2-II, two clones of HL60 cells that had been transfected with the pRc-RSV vector that contained cDNA for human *bcl-2*. (B) Fractionation by agarose gel electrophoresis of DNA from HL60/neo and HL60/bcl-2-II cells that had been treated with 6×10^{-9} M (lane 2), 9×10^{-9} M (lane 3), or 3×10^{-8} M (lane 4) bufalin for 24 h. Lane 1 shows control. (C) The extent (%) of DNA fragmentation was determined using DAPI. HL60/neo and two clones of HL60/bcl-2 cells were treated with bufalin at various concentrations for 24 h. (D) Changes in the plasma membrane potential of HL60/neo and HL60/bcl-2-II cells that had been treated with 10^{-6} M bufalin for the indicated times. The plasma membrane potential was analyzed by flow cytometry using rhodamine 123 and is expressed in terms of the intensity of fluorescence of rhodamine 123 incorporated into cells that comprised the largest population in each sample. (C and D) Data presented are the means ± SD of results from three independent experiments. Absence of bars indicates that the SD was insignificant. (E) Release of cytochrome *c* in HL60/bcl-2-II cells that had been treated with bufalin were measured by immunoblotting with antibodies against cytochrome *c*. Lanes 1, 3 and 5, untreated; lanes 2, 4 and 6, treated with 9×10^{-9} M bufalin for 24 h.

induction of DNA fragmentation by bufalin. Figure 7D shows the effects of bufalin on the plasma membrane potential of HL60/neo and HL60/bcl-2-II cells, as analyzed by flow cytometry after staining with rhodamine 123. The membrane potential of HL60/neo cells was markedly decreased by treatment with 10^{-6} M bufalin for 3 h, as well as for 6 h. The membrane potential of HL60/bcl-2 cells was also markedly reduced by treatment with 10^{-6} M bufalin for 3 and 6 h. The results indicate that the Na^+,K^+-ATPase of HL60/bcl-2 cells was inhibited by bufalin even though the induction by bufalin of DNA fragmentation in these cells was markedly suppressed, as shown in Fig. 7, B and C. In order to examine whether that overexpression of *bcl-2* may inhibit release of cytochrome *c* in the apoptotic process induced by bufalin, bcl-2-overexpressed HL60/bcl-2 cells were treated with bufalin. As shown in Fig. 7E, the cytochrome *c* was released from mitochondria by treatment of HL60/WT and HL60/neo cells with 9×10^{-9} M bufalin for 24 h, whereas no release of cytochrome *c* was observed in HL60/bcl-2-II cells by the same treatment.

DISCUSSION

In this study, we investigated the effects of bufalin on the induction of apoptosis in various lines of cells. Bufalin induced apoptosis in human tumor cells, such as leukemia THP-1, leukemia MOLT-3 and colon COLO320DM cells, but not in human normal peripheral leukocytes. It is possible, therefore, that bufalin or its derivatives might be a useful chemotherapeutic agent for the treatment of human cancers.

Bufalin did not induce apoptosis in murine leukemia P388D1 and M1 cells. Thus, bufalin seemed effective for human tumor cells but not for murine tumor cells. In the present study, we examined the differences in sensitivity to bufalin among human tumor cells, murine tumor cells, and human normal cells by measuring changes in the membrane potential, which is maintained by the Na^+,K^+-ATPase that catalyzes the coupled active transport of Na^+ and K^+ ions across the plasma membrane. The electric potential difference (E) across the plasma membranes of cells is a function of the concentrations of K^+ ions inside and outside cells, as shown in Eq. 1:

$$E = -2.3\ (RT/F)\ \log_{10}\ [K^+]_c/[K^+]_{out} \qquad (1)$$

where $[K^+]_c$ and $[K^+]_{out}$ are the concentrations of K^+ ions in the cytosol and in the medium, respectively. *R*, *T*, and *F* are thermodynamic constants. When the Na^+,K^+-ATPase is inhibited, the concentration of K^+ ions in the cytosol falls with a resultant decrease in membrane potential. Since the uptake of a cationic dye is dependent on the membrane potential, inhibition of Na^+,K^+-ATPase induces a decrease in the uptake of rhodamine 123 by cells, resulting in a decrease in the fluorescence emitted from inside the cells. Our flow cytometry analysis indicated that the plasma membrane potential of human tumor cells was decreased by treatment with bufalin, while no changes were observed in murine P388D1 cells. Since a decrease in the plasma membrane potential reflects the inhibition of the Na^+,K^+-ATPase on the plasma membrane of cells, our findings indicate that bufalin inhibited the Na^+,K^+-ATPase activity of human tumor cells but not that of murine tumor cells. Indeed, Na^+,K^+-ATPase prepared from murine leukemia M1 cells has been shown to be insensitive to bufalin (*15*). The inhibitory effect of bufalin on the Na^+,K^+-ATPase is well correlated with the drug's capacity to induce apoptosis in human tumor cells. The first target of bufalin is considered to be the Na^+,K^+-ATPase (*25*). Thus, inhibition of this enzyme by bufalin might result in an increase in the level of intracellular Na^+ ions and lead to the induction of apoptosis. Ouabain, a specific inhibitor of Na^+,K^+-ATPases, also induced apoptosis in HL60 cells (data not shown). Moreover, the cytotoxic effects of bufalin were not observed in low-sodium RPMI-1640 medium, where Na^+ ions were replaced by choline (*26*). These results suggest that an increase in the intracellular concentration of Na^+ ions might be important in the induction of apoptosis by bufalin. Thus, our observation that human normal peripheral leukocytes and murine leukemia cells were unaffected by bufalin might be explained by differences between the molecular structures of the Na^+,K^+-ATPase of normal leukocytes and that of tumor cells. Different isozymes are expressed in different cell types and tissues (*27*). Alternatively, a signal transduction pathway from the Na^+,K^+-ATPase to the induction of apoptosis might not exist in normal leukocytes.

We used the uptake of rhodamine 123 to monitor changes in the membrane potential of cells. Since this compound is absorbed by mitochondria, a change in the membrane potential of mitochondria might affect such measurements of plasma membrane potential. We, therefore, examined the membrane potential of mitochondria using JC-1, which has been reported to allow measurements of mitochondrial membrane potential specifically (*28*). Using JC-1, we detected no significant changes in mitochondrial membrane potential within 6 h of the start of treatment with bufalin (data not shown). Moreover, treatment with bufalin did not cause any morphological changes in tumor cells, such as THP-1 and P388D1 cells, within 6 h, as judged from the front scattering of cells in suspension by flow cytometry (data not shown). It is, therefore, reasonable to assume that the changes in rhodamine fluorescence observed within 6 h (Fig. 4C) were attributable to changes in the plasma membrane potential of cells. However, we cannot exclude the possibility that damage to mitochondria might occur after a change in plasma membrane potential has been induced by bufalin. In fact, at 9 h, we detected the release of cytochrome *c* from mitochondria in THP-1 cells that had been treated with bufalin (Fig. 6A). The release of cytochrome *c* from mitochondria occurs upon changes in mitochondrial membrane potential and has been reported to lead to the induction of apoptosis (*29, 30*). In dexamethasone-treated thymocytes, mitochondria are functionally intact during the early stages of apoptosis, but depolarization of the mitochondrial membrane and a decrease in the mass of mitochondria occur after DNA fragmentation (*28*). Taken together, our results suggest that the first target of bufalin might be the plasma membrane Na^+,K^+-ATPase, with much later release of cytochrome *c* from mitochondria leads, in turn, to the activation of caspases.

It has been suggested that oncogenes, such as *bcl-2*, *c-myc*, and *p53*, might be involved in the induction of apoptosis in various cells (*31-34*). Overexpression of the *bcl-2* gene inhibits the induction of apoptosis in leukemia cells (*35*) and renders cells resistant to apoptosis (*36, 37*). The transcriptional factor c-myc is involved not only in apoptosis but also in the proliferation and differentiation of

cells (*32*). Enhanced expression of the c-*myc* gene promotes cell proliferation, while down-regulation leads to differentiation (*38*). Evan *et al.* (*39*) demonstrated that expression of c-*myc* leads to apoptosis only when cells are deprived of growth factors. Expression of p53 protein leads to activation of c-Myc and induces apoptosis in quiescent fibroblasts (*40*). In the present study, overexpression of *bcl-2* prevented bufalin-induced apoptosis as well as release of cytochrome *c* from mitochondria but did not prevent the inhibition of the Na^+,K^+-ATPase by bufalin. In a previous study, we showed that induction of apoptosis by bufalin was correlated with a decrease in the level of expression of the *bcl-2* gene in HL60 cells (*13*), suggesting the involvement of down-regulation of the *bcl-2* gene in bufalin-induced apoptosis. Thus, we propose that Na^+,K^+-ATPase acts upstream of bcl-2, and inhibition of the Na^+,K^+-ATPase by bufalin down-regulates expression of the *bcl-2* gene with resultant induction of apoptosis. Our finding that HL60/bcl-2 cells that overexpressed *bcl-2* were resistant to the bufalin-induced fragmentation of DNA but still exhibited a change in plasma membrane potential in response to bufalin suggests that the apoptosis-inducing signal from the Na^+,K^+-ATPase in HL60/bcl-2 cells cannot be transduced. It is unknown how the bufalin signal is transmitted from the Na^+,K^+-ATPase to influence the expression of the *bcl-2* gene. Further work is needed to clarify the signal transduction pathway that follows the exposure of cells to bufalin.

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REFERENCES

1. Kerr, J.F.R., Wyllie, A.H., and Currie, A.R. (1972) Apoptosis: abasic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**, 239-257
2. Pierce, G.B., Lewellyn, A.L., and Parchment, R.E. (1989) Mechanism of programmed cell death in the blastocyst. *Proc. Natl. Acad. Sci. USA* **86**, 3654-3658
3. Vaux, D.L., Cory, S., and Adams, J.M. (1988) *Bcl-2* gene promotes haemopoietic cell survival and cooperates with c-*myc* to immortalize pre-B cells. *Nature* **335**, 440-442
4. Leonardo, M.J. (1991) Interleukin-2 programs mouse $\alpha\beta$ T lymphocytes for apoptosis. *Nature* **353**, 858-861
5. Ucker, D.S. (1987) Cytotoxic T lymphocytes and glucocorticoids activate an endogenous suicide process in target cells. *Nature* **327**, 62-64
6. Laurent-Crawford, A.G., Krust, B., Muller, S., Riviere, Y., Rey-Cuille, M.A., Bechet, J.M., Montagnier, L., and Hovanessian, A.G. (1991) The cytopathic effect of HIV is associated with apoptosis. *Virology* **185**, 829-839
7. Wyllie, A.H., Morris, R.G., Smith, A.L., and Dunlop, D. (1984) Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J. Pathol.* **142**, 67-77
8. Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E., and Croce, C.M. (1985) The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. *Science* **229**, 1390-1393
9. Bakhshi, A., Jensen, J.P., Goldman, P., Wright, J.J., McBride, O.W., Epstein, A.L., and Korsmeyer, S.J. (1985) Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcription unit on chromosome 18. *Cell* **41**, 899-906
10. Nunez, G., London, L., Hockenbery, D., Alexander, M., Mckearn, J.P., and Korsmeyer, S.J. (1990) Deregulated *bcl-2* gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines. *J. Immunol.* **144**, 3602-3610
11. McDonnell, T.J., Deane, N., Platt, F.M., Nunez, G., Jaeger, U., Mckearn, J.P., and Korsmeyer, S.J. (1989) *Bcl-2*-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. *Cell* **57**, 79-88
12. Strasser, A., Harris, A.W., and Cory, S. (1991) *Bcl-2* transgene inhibits T cell death and perturbs thymic self-censorship. *Cell* **67**, 889-899
13. Masuda, Y., Kawazoe, N., Nakajo, S., Yoshida, T., Kuroiwa, Y., and Nakaya, K. (1995) Bufalin induces apoptosis and influences the expression of apoptosis-related genes in human leukemia cells. *Leukemia Res.* **19**, 549-556
14. Watabe, M., Masuda, Y., Nakajo, S., Yoshida, T., Kuroiwa, Y., and Nakaya, K. (1996) The cooperative interaction of two different signaling pathways in response to bufalin induces apoptosis in human leukemia U937 cells. *J. Biol. Chem.* **271**, 14067-14073
15. Numazawa, S., Shinoki, M., Ito, H., Yoshida, T., and Kuroiwa, Y. (1994) Involvement of Na^+,K^+-ATPase inhibition in K562 cell differentiation induced by bufalin. *J. Cell. Physiol.* **160**, 113-120
16. Unten, S., Sakagami, H., and Konno, K. (1989) Stimulation of granulocytic cell iodination by pine cone antitumor substances. *J. Leuk. Biol.* **45**, 168-175
17. Martin, S.J., Lennon, S.V., Bonham, A.M., and Cotter, T.G. (1990) Induction of apoptosis (programmed cell death) in human leukemic HL-60 cells by inhibition of RNA or protein synthesis. *J. Immunol.* **145**, 1859-1867
18. Wyllie, A.H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284**, 555-556
19. Brunk, C.F., Jones, K.C., and James, T.W. (1979) Assay for nanogram quantities of DNA in cellular homogenates. *Anal. Biochem.* **92**, 497-500
20. Davis, S., Weiss, M.J., Wong, J.R., Lampidis, T.J., and Chen, L.B. (1985) Mitochondrial and plasma membrane potentials cause unusual accumulation and retention of rhodamine 123 by human breast adenocarcinoma-derived MCF-7 cells. *J. Biol. Chem.* **260**, 13844-13850
21. Noguchi, K., Nakajima, M., Naito, M., and Tsuruo, T. (1995) Inhibition by differentiation-inducing agents of wild-type p53-dependent apoptosis in HL-60 cells. *Jpn. J. Cancer Res.* **86**, 217-223
22. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159
23. Maniatis, T., Fritsch, E.F., and Sambrook, J. (eds.) (1982) *Molecular Cloning: A Laboratory Manual.*, pp. 188-206, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Smiley, S.T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T.W., Steele, G.D., and Chen, L.B. (1991) Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. *Proc. Natl. Acad. Sci. USA* **88**, 3671-3675
25. Zhang, L.S., Nakaya, K., Yoshida, T., and Kuroiwa, Y. (1992) Induction by bufalin of differentiation of human leukemia cells HL60, U937, and ML1 toward macrophage/monocyte-like cells and its potent synergistic effect on the differentiation of human leukemia cells in combination with other inducers. *Cancer Res.* **52**, 4634-4641
26. Numazawa, S., Inoue, N., Nakura, H., Sugiyama, T., Fujino, E., Shinoki, M., Yoshida, T., and Kuroiwa, Y. (1996) A cardiotonic steroid bufalin-induced differentiation of THP-1 cells. *Biochem. Pharmacol.* **52**, 321-329
27. Sweadner, K.J. (1989) Isozymes of the Na^+/K^+-ATPase. *Biochim. Biophys. Acta* **988**, 185-220
28. Cossarizza, A., Kalashnikova, G., Granssilli, E., Chiappelli, F., Salvioli, S., Capri, M., Barbieri, D., Troiano, L., Monti, D., and Franceschi, C. (1994) Mitochondrial modifications during rat thymocyte apoptosis: a study at the single cell level. *Exp. Cell. Res.* **214**, 323-330
29. Liu, X., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X. (1996) Induction of apoptotic program in cell-free extracts:

requirement for dATP and cytochrome *c*. *Cell* **86**, 147-157
30. Shimizu, S., Eguchi, Y., Kamiike, W., Waguri, S., Uchiyama, Y., Matsuda, H., and Tsujimoto, Y. (1996) Bcl-2 blocks loss of mitochondrial membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory chain inhibitors. *Oncogene* **13**, 21-29
31. Hockenbery, D.M., Nunez, G., Milliman, C., Schreiber, R.D., and Korsmeyer, S.J. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* **348**, 334-336
32. Askew, D.S., Ashmun, R.A., Simmons, B.C., and Cleveland, J.L. (1991) Constitutive c-*myc* expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* **6**, 1915-1922
33. Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A., and Oren, M. (1991) Wild-type *p53* induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6. *Nature* **352**, 345-347
34. Fanidi, A., Harrington, E.A., and Evan, G.I. (1992) Cooperative interaction between c-*myc* and *bcl-2* proto-oncogenes. *Nature* **359**, 554-556
35. Miyashita, T. and Reed, J.C. (1993) Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood* **81**, 151-157
36. Hockenbery, D.M., Zutter, M., Hickey, W., Nahm, M., and Korsmeyer, S.J. (1991) Bcl2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proc. Natl. Acad. Sci. USA* **88**, 6961-6965
37. Lebrun, D.P., Warnke, R.A., and Cleary, M.L. (1993) Expression of *bcl-2* in fetal tissues suggests a role in morphogenesis. *Am. J. Pathol.* **142**, 743-753
38. Kelly, K. (1986) The regulation and expression of *c-myc* in nomal and malignant cells. *Annu. Rev. Immunol.* **4**, 317-338
39. Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z., and Hancock, D.C. (1992) Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69**, 119-128
40. Hermeking, H. and Eick, D. (1994) Mediation of c-Myc-induced apoptosis by p53. *Science* **265**, 2091-2093