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# Differential Induction of Apoptosis in B16 Melanoma and EL-4 Lymphoma Cells by Cytostatin and Bactobolin

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**Most solid tumor cells are less sensitive to apoptosis induced by anticancer drugs than hematopoietic cancer cells. However, the mechanisms of the different responses to apoptosis in these cell types remain unknown. To explore this question, we used B16 melanoma and EL-4 lymphoma cells as solid tumor- and hematopoietic cancer-derived cell lines, and examined the effects of two apoptosis inducers, cytostatin and bactobolin, on both cell lines. Apoptosis in B16 cells was induced strongly by bactobolin, but weakly by cytostatin. In contrast, apoptosis in EL-4 cells was induced strongly by cytostatin, but weakly by bactobolin. While caspase-3 was activated upon induction of apoptosis in both cell lines, Ac-DEVD-CHO, a specific inhibitor of caspase-3, suppressed only the apoptosis in B16 cells. In B16 cells, cyclins E, A, and B1 were decreased by strongly apoptosis-inducing bactobolin prior to apoptosis commitment, but cyclin E was not decreased by weakly apoptosis-inducing cytostatin. On the other hand, in EL-4 cells cyclins D1, E, A, and B1 were decreased by strongly apoptosis-inducing cytostatin prior to apoptosis commitment, but neither cyclin A nor B1 was decreased by weakly apoptosis-inducing bactobolin. These results indicate that the dependency of apoptosis induction on caspase activity is different between the two cell lines. Furthermore, there may be an inverse correlation between specific cyclins and apoptosis induction in the two cell lines.**

Key words: Solid tumor — Apoptosis — Cytostatin — Bactobolin — Cyclin

The evidence that many anticancer drugs induce apoptosis in various cancer cells *in vitro* supports the idea that they also act as apoptosis inducers *in vivo*.[1–4] Therefore, induction of apoptosis specifically in cancer cells is one possible approach for cancer chemotherapy. However, among cancer cell lines used *in vitro*, hematopoietic cancer cells such as leukemias and lymphomas are generally more sensitive to apoptosis induced by anticancer drugs than solid tumor cells.[5] This phenomenon is consistent with the poor efficacy of chemotherapy for solid tumors. More powerful and selective apoptosis inducers for solid tumor cells seem to be needed.

A family of intracellular cysteine proteases, the caspases, may play an important role in apoptosis induced by various stimuli. Fas-induced apoptosis requires the sequential activation of caspase-1 (ICE) and caspase-3 (CPP32).[6] Various anticancer drugs are also reported to induce apoptosis in cancer cells, accompanied with activation of caspase-3.[5, 7] When activated, the caspases cleave a variety of intracellular substrates such as poly(ADP-ribose)polymerase (PARP).[7, 8] While Bcl-2 and Bcl-$X_L$ suppress apoptosis by inhibiting the activation of caspases, Bax and Bcl-$X_S$ promote apoptosis.[9–12] Thus, many molecules have been reported to be involved in the apoptotic pathway. However, mechanisms underlying the different sensitivities of solid tumor and hematopoietic cancer cells to apoptosis induction remain unknown. Identification of a selective pathway or target for apoptosis induction in solid tumor cells would be helpful for the development of new anticancer drugs.

In this study, we used B16 melanoma and EL-4 lymphoma cells as solid tumor- and hematopoietic cancer-derived cell lines, respectively and two apoptosis inducers, cytostatin and bactobolin. Cytostatin was first isolated as an inhibitor of cell adhesion to extracellular matrix components and was found to inhibit experimental metastasis *in vivo*.[13–15] Bactobolin was isolated as an antitumor compound and shown to suppress tumor growth *in vivo*.[16, 17] We have recently found that both cytostatin and bactobolin induce apoptosis in some cell lines.[18] Using these two drugs and comparing the responsiveness of B16 and EL-4 cells to apoptosis induction, we found that the sensitivities and the possible mechanism of apoptosis induction were completely different between the two cell lines.

## MATERIALS AND METHODS

**Reagents and antibodies** Cytostatin and bactobolin were prepared as described.[13, 16] Specific inhibitors of caspase-1 and caspase-3, Ac-YVAD-CHO and Ac-DEVD-CHO, respectively, and fluorogenic substrates for caspase-1 and caspase-3, YVAD-MCA and DEVD-MCA, respectively, were purchased from the Peptide Institute (Osaka). Antibodies used for western blotting were the following: anti-

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Bax (sc-526), anti-cyclin A (sc-596), and anti-cyclin B1 (sc-245) were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Bcl-2 (B46620) and anti-Bcl-x (B22630) were from Transduction Laboratories (Lexington, KY); anti-cyclin D1 (05-362) and anti-cyclin E (06-459) were from Upstate Biotechnology (Lake Placid, NY).

**Cells** Mouse B16 melanoma and mouse EL-4 T lymphoma cells were maintained in Dulbecco's modified Eagle's medium and RPMI1640 medium, respectively, supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS), 100 units/ml of penicillin G, and 100 $\mu$g/ml of streptomycin at 37°C with 5% $CO_2$.

**DNA fragmentation** Treated cells ($3\times10^5$) were washed with phosphate-buffered saline (PBS) and lysed in 100 $\mu$l of lysis buffer containing 0.5% Triton X-100, 10 m*M* Tris-HCl (pH 7.4), and 10 m*M* EDTA at room temperature for 10 min. The supernatant fractions were collected by centrifugation at 15,000 rpm for 10 min and treated with RNase A (Sigma, St. Louis, MO) at 37°C for 1 h, and then with proteinase K (GIBCO-BRL, Gaithersburg, MD) at 37°C for 1 h. The DNA in these fractions was precipitated overnight with 20 $\mu$l of 5 *M* NaCl and 120 $\mu$l of 2-isopropanol at −20°C. The DNA was dissolved in 10 $\mu$l of 10 m*M* Tris-HCl (pH 7.4) and 1 m*M* EDTA buffer. The DNA samples were electrophoretically separated on 1.2% agarose gels. After electrophoresis at 100 V for 1 h, DNA was visualized by ethidium bromide staining.

**Cytotoxicity** Cells were inoculated in 96-well plates at 5000 cells/well and incubated with or without test drugs for 3 days. The growth was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described.[19]

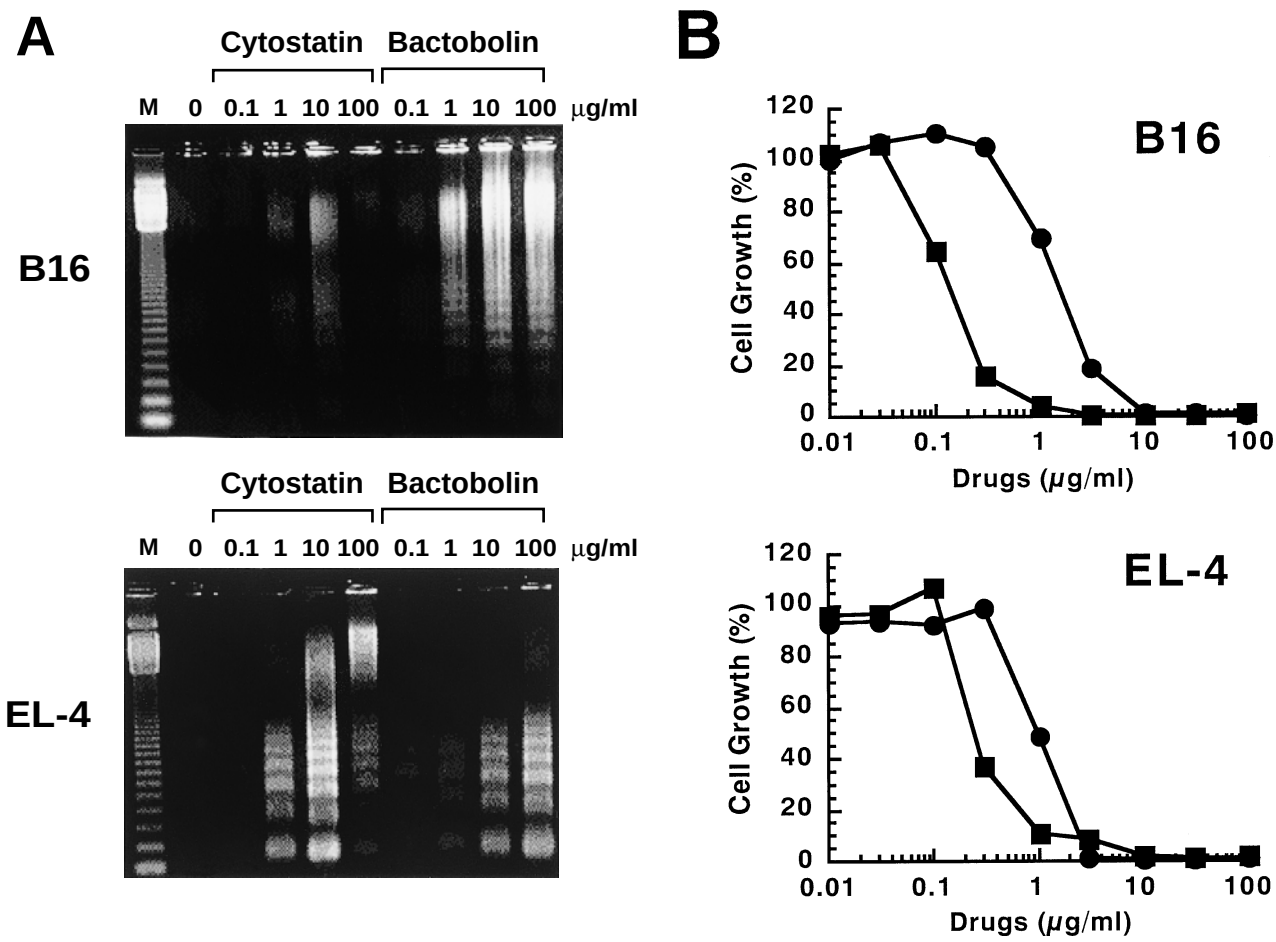


Fig. 1. Effect of cytostatin and bactobolin on B16 and EL-4 cells. (A) B16 and EL-4 cells were treated with the indicated concentrations of cytostatin or bactobolin for 24 h (B16 cells) or 6 h (EL-4 cells). Fragmented DNA was isolated and electrophoresed. M, 123 bp DNA ladder marker. (B) B16 and EL-4 cells were treated with the indicated concentrations of cytostatin (●) or bactobolin (■) for 3 days. Cell growth was determined using MTT. The values are means of 3 independent duplicate determinations. Each SE is less than 10%.

**Caspase activity** The cell lysates were prepared as described above. Protein concentrations were adjusted and equal protein amounts of lysates were incubated with 50 $\mu M$ YVAD-MCA or DEVD-MCA at 37°C for 30 min. The release of amino-4-methylcoumarin was monitored by a spectrofluorometer (Labsystems Fluoroskan II; Dainippon Seiyaku, Osaka) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

**Preparation of cell lysates and western blotting** Treated cells ($3\times10^5$) were washed twice with ice-cold PBS containing 100 $\mu M$ $Na_3VO_4$ and then lysed in a lysis buffer (20 m$M$ Hepes [pH 7.5], 150 m$M$ NaCl, 1% Triton X-100, 10% glycerol, 1 m$M$ EDTA, 50 m$M$ NaF, 50 m$M$ β-glycerophosphate, 1 m$M$ $Na_3VO_4$, and 25 $\mu$g/ml each of antipain, leupeptin, and pepstatin). Equal amounts of protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Enhanced chemiluminescence (Amersham, Arlington Heights, IL) was used to visualize the immunoblot signals.

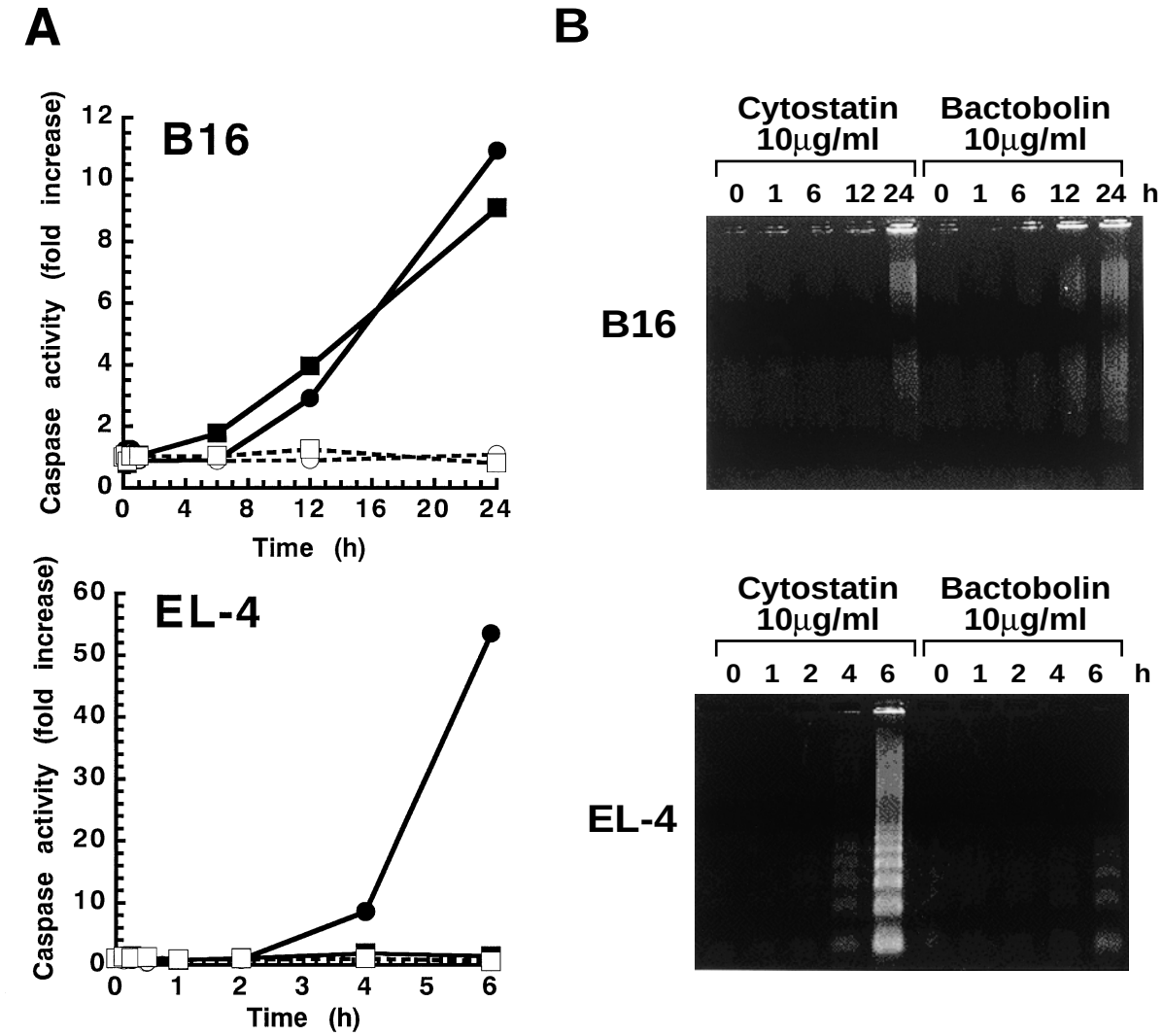


Fig. 2. Effect of cytostatin and bactobolin on caspase activity. (A) B16 and EL-4 cells were treated with 10 $\mu$g/ml of cytostatin (○, ●) or bactobolin (□, ■) for the indicated times. Cell lysates were prepared and activities of caspase-1 (○, □) and caspase-3 (●, ■) were determined as described in "Materials and Methods." The values are means of 3 independent duplicate determinations. Each SE is less than 10%. (B) B16 and EL-4 cells were treated with 10 $\mu$g/ml of cytostatin or bactobolin for the indicated times. DNA fragmentation was assessed as described in "Materials and Methods."

## RESULTS

**Effects of cytostatin and bactobolin on B16 and EL-4 cells** We first examined the effects of cytostatin and bactobolin on apoptosis and growth of B16 and EL-4 cells. Apoptosis induction was assessed in terms of nucleosomal fragmentation of genomic DNA and observed mainly at the minimum required treatment time to study the primary effects of the drugs. In B16 cells, cytostatin induced apoptosis weakly at 10 $\mu$g/ml, whereas bactobolin induced it strongly even at 1 $\mu$g/ml, in 24 h treatment (Fig. 1A). By contrast, in EL-4 cells, bactobolin induced apoptosis weakly even at 100 $\mu$g/ml, but cytostatin induced it strongly at 10 $\mu$g/ml, in just a 6 h treatment (Fig. 1A). The same result was obtained in EL-4 cells upon 24 h treatment (data not shown). Thus, cytostatin and bactobolin induced strong apoptosis preferentially in EL-4 and B16 cells, respectively. On the other hand, cytostatin and bactobolin inhibited B16 cell growth with 50% growth-inhibitory concentration ($IC_{50}$) values of 2 and 0.2 $\mu$g/ml, respectively, and also inhibited EL-4 cell growth with $IC_{50}$ values of 1 and 0.2 $\mu$g/ml, respectively (Fig. 1B). Thus, bactobolin inhibited the growth of both cell lines more strongly than did cytostatin. However, these growth-inhibitory effects did not correlate with the apoptosis-inducing activities. These results, therefore, indicated that the difference in the sensitivity of these cell lines to the drugs does not simply reflect differential apoptosis induction in the cell lines.

**Effects of cytostatin and bactobolin on caspase activity** Many drugs induce apoptosis in tumor cells concomitantly with the activation of caspases.[5, 7] To examine the involvement of caspase activation in the apoptosis in B16 and EL-4 cells, we examined the effects of cytostatin and bactobolin on caspase activities. In B16 cells, caspase-1 activity was not changed by either of the drugs, but caspase-3 activity was increased time-dependently to the same level by cytostatin and bactobolin (Fig. 2A). In EL-4 cells, caspase-1 was not activated by either of the drugs, as in B16 cells, but caspase-3 activity was greatly increased only by cytostatin (Fig. 2A). These activations of caspase-3 were concomitant with DNA fragmentation in both cell lines (Fig. 2B). To confirm the possible involvement of caspase-3 activation in apoptosis induction, we examined the effect of specific inhibitors of caspase-1 and caspase-3, Ac-YVAD-CHO and Ac-DEVD-CHO, respectively, on the apoptosis. In B16 cells bacto-

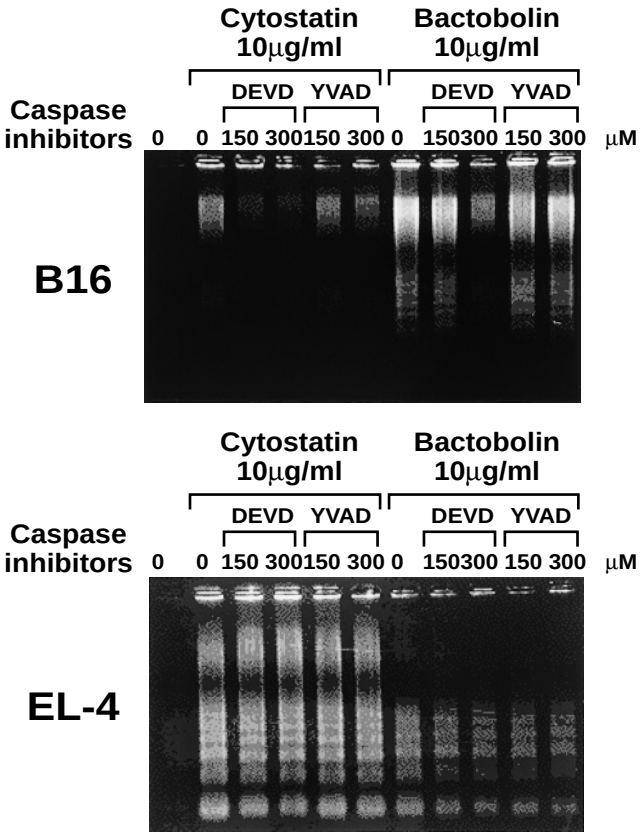


Fig. 3. Effect of caspase inhibitors on cytostatin- and bactobolin-induced apoptosis. B16 and EL-4 cells were pretreated with the indicated concentrations of Ac-DEVD-CHO (DEVD) or Ac-YVAD-CHO (YVAD) for 1 h. Then, the cells were further treated with 10 $\mu$g/ml of cytostatin or bactobolin for 24 h (B16 cells) or 6 h (EL-4 cells). DNA fragmentation was assessed as described in "Materials and Methods."

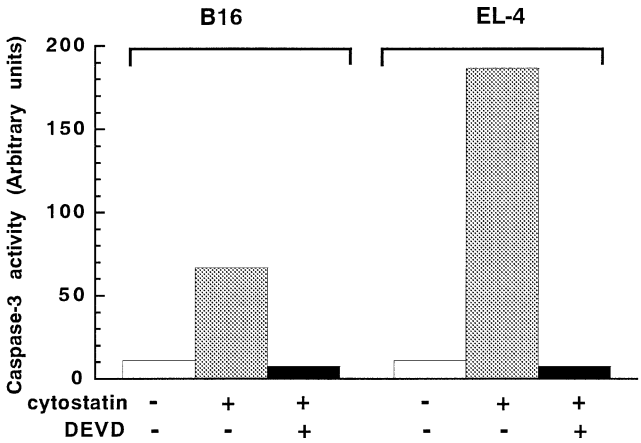


Fig. 4. Effect of caspase-3 inhibitor on cytostatin-induced caspase-3 activation. B16 and EL-4 cells were pretreated with (■) or without (□, ▨) 300 $\mu M$ Ac-DEVD-CHO (DEVD) for 1 h and then further treated with 10 $\mu$g/ml of cytostatin for 24 h (B16 cells) or 6 h (EL-4 cells) (▨, ■). Caspase-3 activity was assessed as described in "Materials and Methods." The values are means of 3 independent duplicate determinations. Each SE is less than 10%.

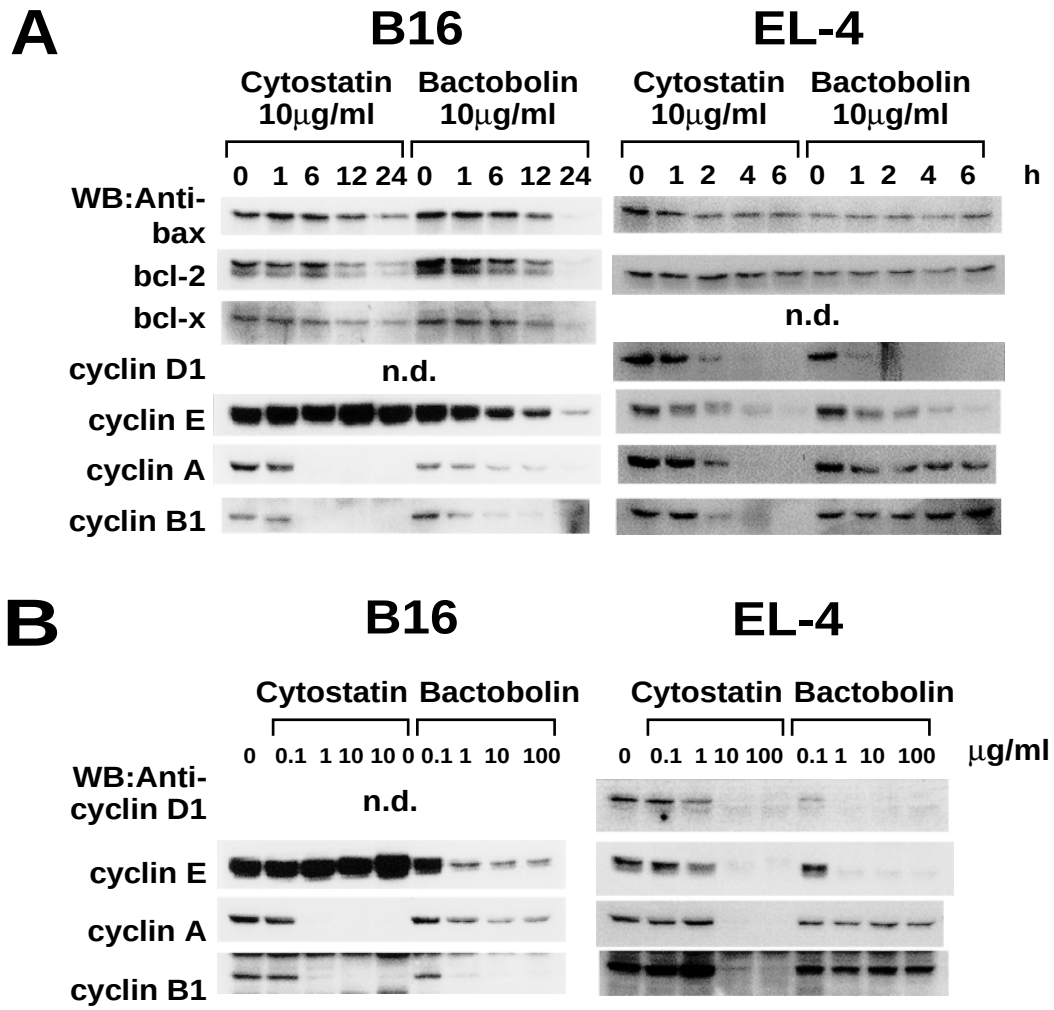


Fig. 5. Effect of cytostatin and bactobolin on apoptosis- and cell cycle-related molecules. B16 and EL-4 cells were treated with 10 $\mu$g/ml of cytostatin or bactobolin for the indicated times (A) or treated with the indicated concentrations of cytostatin or bactobolin for 24 h (B16 cells) or 6 h (EL-4 cells) (B). Cell lysates were prepared and western blotting was done using the indicated antibodies. n.d., not detectable.

bolin-induced strong apoptosis was inhibited by Ac-DEVD-CHO, but not by Ac-YVAD-CHO (Fig. 3). Furthermore, cytostatin-induced weak apoptosis in B16 cells was also inhibited by Ac-DEVD-CHO (Fig. 3). By contrast, in EL-4 cells cytostatin-induced strong apoptosis was not inhibited by Ac-DEVD-CHO or Ac-YVAD-CHO (Fig. 3). However, cytostatin-induced activation of caspase-3 was inhibited in Ac-DEVD-CHO-treated EL-4 cells as well as B16 cells (Fig. 4). Therefore, these results suggested that apoptosis in B16 cells was dependent on caspase-3 activation, but that in EL-4 cells was not.

**Effects of cytostatin and bactobolin on apoptosis- and cell cycle-related molecules** Many molecules such as Bcl-2 and Bax are reported to be involved in apoptotic pathways.[9–12] Cell cycle regulation was also considered to participate in the regulation of cell death.[20] Next, we examined the effect of cytostatin and bactobolin on various apoptosis- and cell cycle-related molecules. In B16 cells both cytostatin and bactobolin at 10 $\mu$g/ml equally decreased Bcl-2 and Bcl-xL time-dependently (Fig. 5A). Unexpectedly, Bax, an apoptotic molecule, was also decreased by both drugs (Fig. 5A). On the other hand, neither cytostatin nor bactobolin affected the amounts of Bax and Bcl-2 in EL-4 cells. These results suggested the possible involvement of Bcl-2 and Bcl-xL in B16 apoptosis, but not in EL-4 apoptosis. As regards cell cycle-related molecules, in B16 cells strongly apoptosis-inducing bactobolin reduced cyclins E, A, and B1 prior to

DNA fragmentation and caspase-3 activation (Figs. 2B and 5A). However, weakly apoptosis-inducing cytostatin rapidly decreased cyclins A and B1, but not cyclin E, even at 100 $\mu$g/ml, in B16 cells (Fig. 5). In contrast, in EL-4 cells, while strongly apoptosis-inducing cytostatin decreased cyclins D1, E, A, and B1 prior to DNA fragmentation and caspase-3 activation (Figs. 2B and 5), weakly apoptosis-inducing bactobolin decreased cyclins D1 and E, but not cyclins A and B even at 100 $\mu$g/ml (Fig. 5). Thus, all cyclins were decreased when apoptosis was strongly induced in both cell lines. However, some specific cyclins were not affected when apoptosis was weakly induced in these cell lines.

## DISCUSSION

In general, solid tumor cells are less sensitive to apoptosis induced by anticancer drugs than are hematopoietic cancer cells.[5] Hematopoietic cells can grow without substrate attachment *in vitro*, but when the cells adhere to a firm solid support such as a plastic dish, their growth tends to be reduced.[21] On the other hand, epithelial cells, from which most solid tumors are derived, cease cell growth in the absence of substrate attachment.[22, 23] Although most solid tumor cells are anchorage-independent, their growth ability also tends to be reduced in suspension culture.[24] Thus, the mechanisms of growth regulations are considered to be basically different in the two cell types. In this study we showed that B16 and EL-4 cells responded differently to the same drug for apoptosis induction. Apoptosis of B16 cells was induced strongly by bactobolin, but weakly by cytostatin. In contrast, apoptosis of EL-4 cells was induced strongly by cytostatin, but weakly by bactobolin (Fig. 1). Although significant growth inhibition by the less apoptosis-inducing drug in each cell line was observed, how such cell death occurred without DNA fragmentation is unknown. However, there apparently exist modes of cell deaths with and without accompanying DNA fragmentation. On the other hand, many anticancer drugs induce apoptosis via activation of caspase, and a specific inhibitor of caspase suppresses the apoptosis.[5, 7] In B16 cells both cytostatin and bactobolin increased caspase-3 activity and Ac-DEVD-CHO, a specific inhibitor of caspase-3, suppressed apoptosis induced by both drugs (Fig. 3). However, cytostatin activated caspase-3 to the same extent as bactobolin, even though cytostatin induced apoptosis to a lesser extent than bactobolin (Fig. 1). Thus, it is suggested that apoptosis in B16 cells was dependent on caspase-3 activation, but activation of caspase-3 alone is not enough for apoptosis induction. On the other hand, in EL-4 cells, strongly apoptosis-inducing cytostatin greatly activated caspase-3, but weakly apoptosis-inducing bactobolin did not (Fig. 2). This result suggested that apoptosis in EL-4 cells was dependent on caspase-3 activation, but Ac-DEVD-CHO did not suppress the cytostatin-induced apoptosis (Fig. 3), even though it inhibited the caspase-3 activation (Fig. 4). Thus, these results suggest that caspase-3 activation is irrelevant to apoptosis induction in EL-4 cells.

To explore the mechanisms of the difference in apoptosis induction between B16 and EL-4 cells, we examined other factors, including apoptosis- and cell cycle-related molecules. In B16 cells, the apoptosis-inhibitory factors Bcl-2 and Bcl-XL were decreased equally by cytostatin and bactobolin (Fig. 5). Unexpectedly, Bax, an apoptosis-inducing factor, was also decreased by both drugs (Fig. 5). The effects of both drugs were almost the same and were correlated with the activation of caspase-3, but not with apoptosis induction. In EL-4 cells, Bax and Bcl-2 were not affected by either of the drugs (Fig. 5). On the other hand, when we assessed the cyclin levels, we found correlations with apoptosis induction. In B16 cells, strongly apoptosis-inducing bactobolin decreased all the cyclins prior to DNA fragmentation, but less apoptosis-inducing cytostatin failed to decrease cyclin E (Figs. 2 and 5). In contrast, strongly apoptosis-inducing cytostatin decreased all cyclins in EL-4 cells prior to DNA fragmentation, but less apoptosis-inducing bactobolin failed to decrease cyclins A and B1 (Figs. 2 and 5). Thus, cells induced to undergo apoptosis showed decreases of all cyclins prior to apoptosis commitment. Cell cycle analysis revealed that apparent cell cycle arrest of B16 and EL-4 cells in the G1, S, or G2/M phases was not induced by cytostatin or bactobolin (data not shown). Thus, the cell cycle arrest did not prevent apoptosis commitment. We speculate that cyclin E and cyclin A and/or B1 suppressed the apoptosis in B16 and EL-4 cells, respectively. To confirm the cyclin regulation of apoptosis, further detailed studies will be needed using other drugs and cell lines. The molecular targets of cytostatin and bactobolin are now being studied, and the precise mechanisms of the different actions on the two cell lines are still unknown. However, it seems possible that modulation of specific cyclins regulates apoptosis selectively in solid tumor cells or hematopoietic cells.

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