Caspase-3 Activation Is Not Responsible for Vinblastine-induced Bcl-2 Phosphorylation and G2/M Arrest in Human Small Cell Lung Carcinoma Ms-1 Cells

Etsu Tashiro,[1] Siro Simizu,[1] Minoru Takada,[2] Kazuo Umezawa[1] and Masaya Imoto[1, 3]

[1]*Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522 and* [2]*Rinku General Medical Center, 2-23 Rinkuohrai-kita, Izumisano-shi, Osaka 598-8577*

Vinblastine arrests cells in the G2/M phase of the cell cycle and subsequently induces cell death by apoptosis. We found that treatment of cells with vinblastine induced phosphorylation of Bcl-2, resulting in the dissociation of Bcl-2 and Bax. Moreover, vinblastine-induced apoptosis was suppressed by an inhibitor of caspase-3, Ac-DEVD-CHO; and a 17-kDa active fragment of caspase-3 was detected following vinblastine treatment, suggesting that caspase-3 is involved in vinblastine-induced apoptosis. However, Ac-DEVD-CHO affected neither vinblastine-induced Bcl-2 phosphorylation nor vinblastine-induced G2/M arrest. Vinblastine caused G2/M arrest prior to apoptosis, whereas vinblastine-induced apoptosis was not dependent on the duration of the G2/M phase. Thus, vinblastine-induced apoptosis might be mediated by the phosphorylation of Bcl-2, resulting in Bcl-2 inactivation, and by subsequent activation of caspase-3.

Key words: Vinblastine — Bcl-2 — Caspase-3 — G2/M arrest

Vinblastine, a vinca alkaloid, is a potent inhibitor of cell proliferation, and induces arrest at the metaphase of mitosis by acting on spindle microtubules in many mammalian cells.[1–3] It is widely used for chemotherapy of lung cancer, breast cancer, and leukemia. It induces apoptosis possibly as a consequence of its inhibitory effect on tubulin polymerization. DNA-damaging anticancer drugs, such as adriamycin, etoposide, camptothecin, and cisplatin, also induce G2/M arrest and apoptosis. A critical regulator of the cellular response to DNA damage is the transcription factor encoded by the *p53* tumor suppressor gene.[4, 5] However, the biochemical mechanism leading to apoptosis in response to the inhibition of tubulin polymerization by vinblastine is still poorly understood.

Several of the biochemical events that contribute to apoptotic cell death have recently been elucidated. Genetic evidence in nematodes, for example, has identified both positive and negative regulators of apoptosis. The key pro-apoptotic gene, *ced-3*, encodes a putative cysteine protease showing sequence similarity to the family of mammalian interleukin-1β-converting enzyme (ICE)-related proteases.[6] Caspase-3 (CPP32/Yama/apopain) is the most widely investigated member of the ICE family. Recent studies have suggested that proteolytic cleavage and activation of caspase-3 may be functionally important in the induction of apoptosis. The immature 32-kDa caspase-3 (pro-caspase-3) is cleaved into a 12-kDa fragment and 17-kDa biologically active caspase-3. Activated caspase-3 cleaves poly(ADP-ribose) polymerase (PARP), an enzyme that responds to DNA damage by polyribosylation of itself and of other substrates.[7–9] In addition, a potent peptide aldehyde inhibitor of caspase-3, Ac-DEVD-CHO, prevented apoptotic events *in vitro*.[8] These data suggest that caspase-3 initiates key events in apoptosis and may be an effector of apoptotic cell death.

Another nematode gene, *ced-9*, which prevents apoptosis, lies genetically upstream of *ced-3* and *ced-4*.[10] The proto-oncogene *bcl-2*, whose *Caenorhabditis elegans* homologue is *ced-9*,[11] can inhibit apoptosis induced by a variety of stimuli. Some of the members of this protein family such as Bax promote apoptosis and antagonize the function of Bcl-2. Bax can form a homodimer or heterodimerize with the death antagonist Bcl-2. The ratio of Bcl-2 to Bax dictates the susceptibility of cells to apoptotic stimuli.[12] Overexpression or stable transfection of Bcl-2 suppressed drug-induced apoptosis in various cells and prevented activation of the caspase cascade.[13–17]

In the present study, we investigated the mechanism of vinblastine-induced apoptosis in human small cell lung carcinoma (SCLC) cells, and found that vinblastine induced the phosphorylation of Bcl-2 in SCLC cells, and phosphorylated Bcl-2 was incapable of forming heterodimers with Bax. In addition, we found caspase-3 to be involved in vinblastine-induced apoptosis in these cells.

MATERIALS AND METHODS

Materials Vinblastine was obtained from Wako Pure Chemical Industries, Ltd. *Escherichia coli* alkaline phosphatase Type III was obtained from Sigma, and Ac-

---

[3] To whom correspondence should be addressed.

DEVD-CHO, from the Peptide Institute (Osaka). Monoclonal anti-Bcl-2 antibody was purchased from DAKO (Glostrup, Denmark), and anti-Bax and anti-CPP32 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture Human small cell lung carcinoma Ms-1 and Ms-13 and human non-small cell lung carcinoma Ma-44 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), kanamycin (0.1 mg/ml), penicillin G (100 units/ml), and L-glutamine (30 mg/liter) at 37°C in 5% CO_2. To synchronize Ms-1 cells, the cells were treated with 2 mM thymidine for 24 h, then the medium was removed, and the cells were washed and exposed to RPMI containing 0.5% FBS. After 14 h, the serum-deficient medium was replaced by RPMI containing 10% FBS, and 1 mM hydroxyurea was added. After the cells had been treated with hydroxyurea for 20 h, the medium was replaced with new medium without hydroxyurea.

Hoechst 33258 staining Cells were fixed with 3% paraformaldehyde for 20 min at room temperature, washed with distilled water, stained with Hoechst 33258 (10 μg/ml) for 5 min, and again washed with distilled water. Nuclear morphology was examined by fluorescence microscopy.

Western blotting Cells were lysed in 200 μl of ice-cold lysis buffer (50 mM Tris, 125 mM NaCl, 0.5% NP-40, 0.1 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride; pH 7.2) for 30 min on ice and centrifuged at 15,000g for 15 min. Loading buffer (42 mM Tris-HCl, 10% glycerol, 2.3% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 0.002% bromophenol blue; pH 6.3) was then added to each lysate, which was subsequently boiled for 2 min and electrophoresed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. Proteins were transferred to a nitrocellulose filter and immunoblotted with appropriate antibodies. Detection was performed with enhanced chemiluminescence reagent (DuPont, Boston, MA).

***In vitro* dephosphorylation** The *in vitro* dephosphorylation assay was performed essentially as described previously.[18] Briefly, 50 μg of cell lysate was incubated with 4 units of *E. coli* alkaline phosphatase at 37°C for 14 h. The dephosphorylation reaction was stopped by adding SDS sample buffer and boiling the mixture for 5 min. The samples were analyzed by western blotting using anti-Bcl-2 antibody.

Flow cytometric analysis of cell-cycle status and apoptosis The cells were washed with phosphate buffered saline (PBS⁻), fixed in ice-cold 70% ethanol, and stored at 4°C. Thereafter, the cells were washed with PBS⁻ and stained with PI solution (0.1 mg/ml propidium iodide, 0.6% NP-40, and 2 mg/ml RNase A in HBSS) for 30 min. The DNA content was determined by flow cytometry (Epics Elite; Coulter, Hialeah, FL).

RESULTS

Bcl-2 phosphorylation by vinblastine in SCLC cells
Vinblastine gradually decreased the viability of Ms-1 cells (Fig. 1A), and increased the number of cells undergoing apoptosis; and at 40 h following exposure to 0.1 μg/ml of vinblastine, the cells displayed condensation of nuclear chromatin and nuclear fragmentation, as demonstrated by Hoechst 33258 staining (Fig. 1B). Next, we examined the effect of vinblastine on the expression of Bcl-2 protein. As shown in Fig. 2A, treatment of Ms-1 cells with 0.1 μg/ml of vinblastine for 15 h resulted in the expression of slower-migrating forms of Bcl-2 proteins. But the slower-
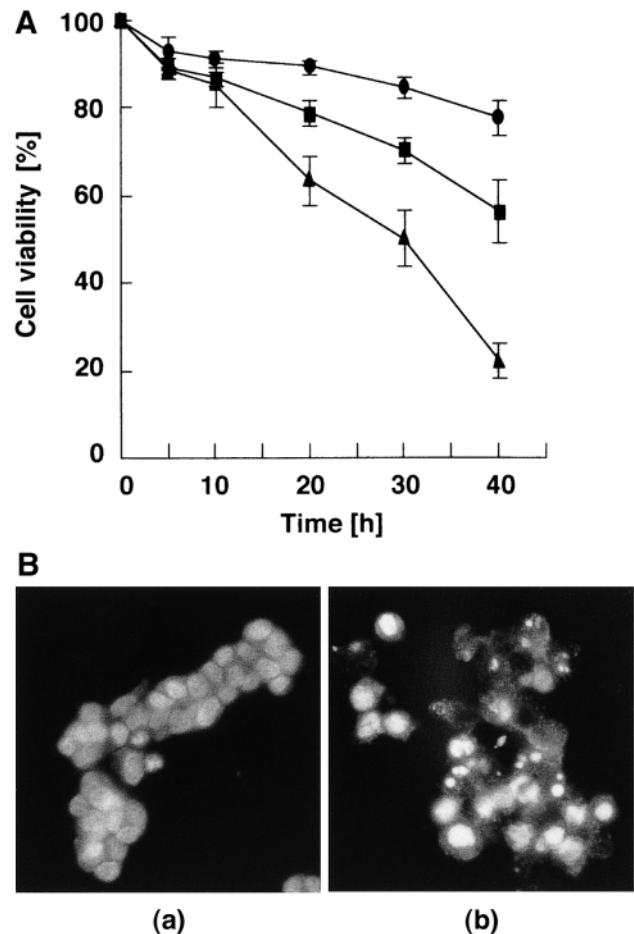

Fig. 1. Induction of apoptosis by vinblastine in Ms-1 cells. (A) Ms-1 cells were treated with 0 (●), 0.01 (■), and 0.1 (▲) μg/ml of vinblastine for the indicated times. Cell viability was assessed by trypan blue dye exclusion assay. (B) Ms-1 cells were left untreated (a) or treated with 0.1 μg/ml of vinblastine for 40 h (b). Then, the cells were stained with Hoechst 33258, observed under a fluorescence microscope and photographed.

migrating form of Bcl-2 was not detected at 0.01 μg/ml of vinblastine treatment under conditions where the cells did not undergo apoptosis (data not shown). The slower-migrating forms of Bcl-2 have been shown by previous studies to represent phosphorylated forms of the protein.[19, 20] To determine whether the slower-migrating form of Bcl-2 protein (30 kDa) in vinblastine-treated cells represented a phosphorylated form of 26-kDa Bcl-2 protein, we incubated vinblastine-treated cell lysates with alkaline phosphatase, as described previously.[21] As shown in Fig. 2A, the slower-migating Bcl-2 (30 kDa) disappeared following the dephosphorylation reaction, suggesting that the mobility change in Bcl-2 induced by the treatment with vinblastine is due to phosphorylation of a portion of the protein. Time-course analysis indicated that the induction of the phosphorylated form of Bcl-2 began at 5 h after the addition of 0.1 μg/ml of vinblastine and that at 10 h, more than 50 % of the Bcl-2 protein was in the phosphorylated form (Fig. 2B). Vinblastine-induced phosphorylation of Bcl-2 protein was also observed in SCLC Ms-13 and non-SCLC Ma-44 cells (data not shown). Moreover, vincristine and taxol, which also interact with tubulin or microtubules, also induced phosphorylation of Bcl-2 protein. In contrast, DNA-damaging anticancer drugs such as adriamycin, etoposide, and cisplatin did not phosphorylate Bcl-2 protein (data not shown)
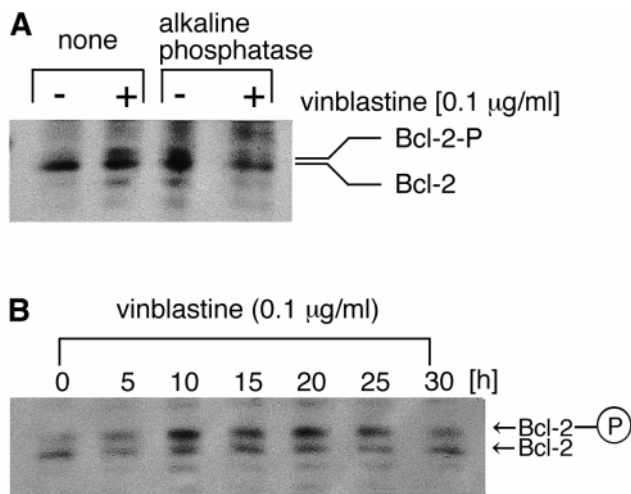
under conditions that result in induction of apoptosis in Ms-1 cells.

Phosphorylated Bcl-2 induced by vinblastine is incapable of forming heterodimers with Bax Previous study has shown that the phosphorylation of Bcl-2 protein disrupts the association of Bcl-2 protein with Bax protein.[19] Therefore, we investigated the effect of vinblastine on the formation of Bcl-2/Bax complexes. Vinblastine-treated Ms-1 cells were collected at various time points, and cell extracts were prepared. Bax/Bcl-2 complex was removed by immunoprecipitation with an excess amount of anti-Bcl-2 antibody, and then the resultant supernatant was subjected to western blotting using anti-Bax antibody. As shown in Fig. 3, the amount of Bax protein in the supernatant, which did not form heterodimers with Bcl-2 protein, increased as Bcl-2 was phosphorylated (Fig. 3 lower panel, cf: Fig. 2B), although the expression level of Bax protein in whole cell extracts was not affected by the vinblastine treatment (Fig. 3 upper panel). These results indicate that vinblastine-induced phosphorylation of Bcl-2 may result in the dissociation of Bcl-2 and Bax, leading to apoptosis.

Caspase-3 was activated by vinblastine Various anticancer drugs have been reported to induce caspase-3 activation in the pathway leading to apoptosis.[16, 17, 22] Therefore, we examined involvement of caspase-3 activation in vinblastine-induced apoptosis using a specific tetrapeptide inhibitor, Ac-DEVD-CHO. When Ms-1 cells preincubated with 100 μM Ac-DEVD-CHO for 2 h were treated with vinblastine, this inhibitor completely blocked apoptosis at least up to 30 h following vinblastine treatment, as determined by the trypan blue dye exclusion assay (Fig. 4A) and by staining with Hoechst 33258 (Fig. 4B). Furthermore, in Ms-1 cells, the amount of 17-kDa
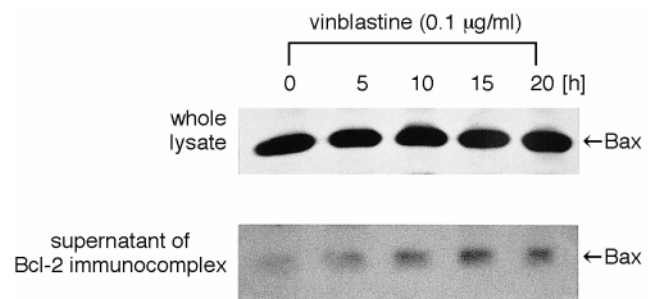

Fig. 2. Induction of Bcl-2 phosphorylation by vinblastine in Ms-1 cells. (A) Ms-1 cells were incubated with or without 0.1 μg/ml of vinblastine for 15 h. Cell lysates were incubated with or without 4 units of alkaline-phosphatase for 14 h at 37°C, and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and western blot analysis with anti-Bcl-2 antibody. (B) Ms-1 cells were treated with 0.1 μg/ml of vinblastine. Samples were then prepared at the indicated times, and analyzed by western blotting using anti-Bcl-2 antibody.

Fig. 3. Effect of Bcl-2 phosphorylation on the formation of Bcl-2/Bax complexes in Ms-1 cells. Ms-1 cells were treated with 0.1 μg/ml of vinblastine. Samples were prepared at the indicated times, and Bcl-2/Bax complexes in the cell lysate were removed by immunoprecipitation with anti-Bcl-2 antibody. Resultant cell lysates or whole cell lysates were subjected to SDS-PAGE and western blot analysis with anti-Bax antibody.
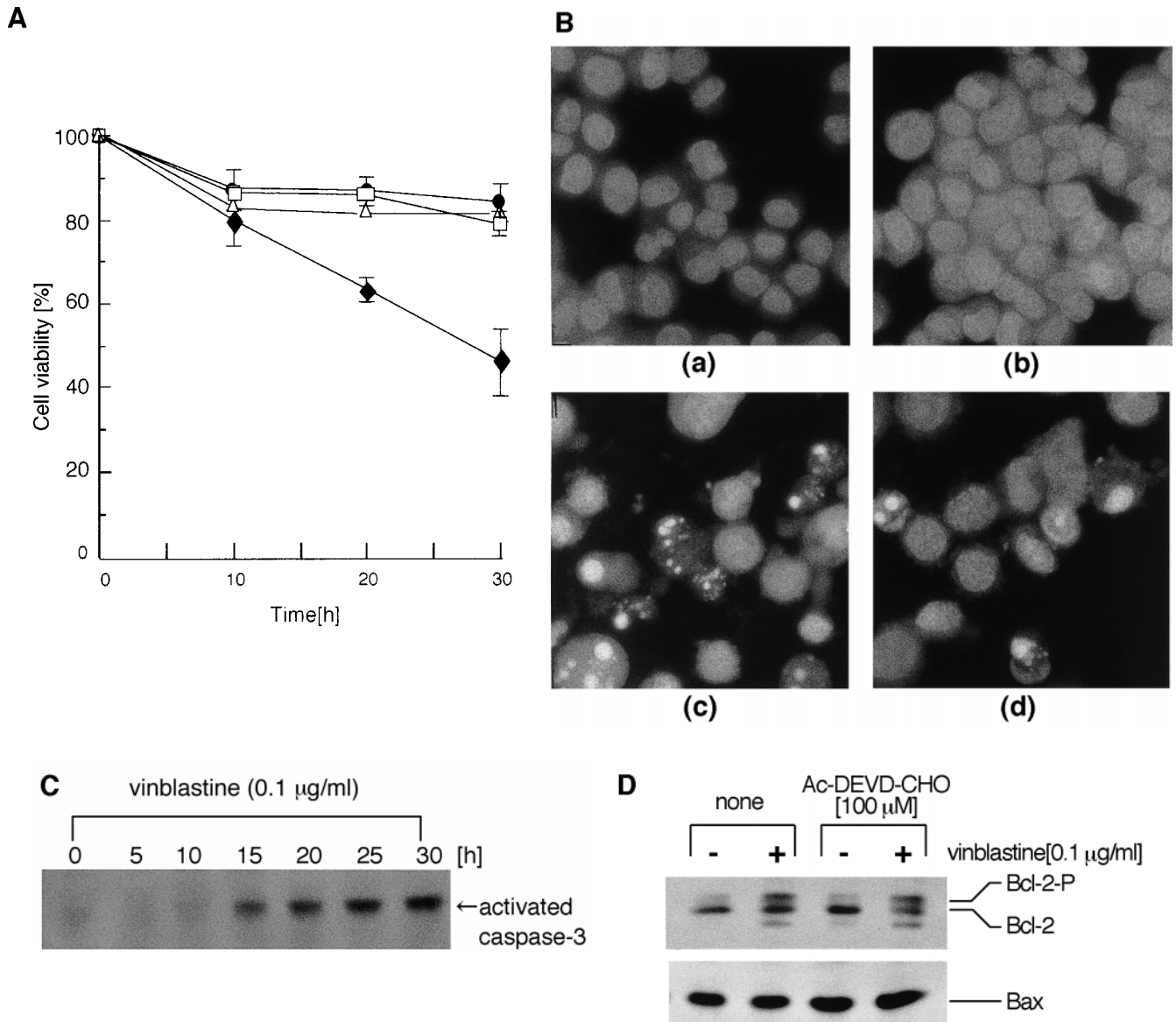
A

B

C

D

Fig. 4. Involvement of caspase-3 activation in vinblastine-induced apoptosis. (A) Suppression of vinblastine-induced cell death. Ms-1 cells were treated without additive (●), or with 0.1 μg/ml of vinblastine (◆), 100 μM Ac-DEVD-CHO (□), or vinblastine and Ac-DEVD-CHO (△) for the indicated times. Cell viability was assessed by trypan blue dye exclusion assay. (B) Suppression of vinblastine-induced apoptosis. Ms-1 cells were treated for 30 h without additive (a), or with 100 μM Ac-DEVD-CHO (b), 0.1 μg/ml of vinblastine (c), or vinblastine and Ac-DEVD-CHO (d). The cells were stained with Hoechst 33258, observed under a fluorescence microscope and photographed. (C) Activation of caspase-3 by vinblastine. Ms-1 cells were treated with 0.1 μg/ml of vinblastine. Samples were prepared at the indicated times, and analyzed by western blotting using anti-CPP32 antibody. (D) Effect of Ac-DEVD-CHO on vinblastine-induced Bcl-2 phosphorylation. Ms-1 cells preincubated with 100 μM Ac-DEVD-CHO for 2 h were treated with 0.1 μg/ml of vinblastine. After 20 h, samples were prepared and analyzed by western blotting using anti-Bcl-2 or anti-Bax antibodies.

caspase-3 active subunit was increased at 15 h after treatment with vinblastine, and maintained at this level up to 24 h (Fig. 4C). These results indicate that caspase-3 activation is required for vinblastine-induced apoptosis. Furthermore, vinblastine induced Bcl-2 phosphorylation prior to causing caspase-3 activation (Figs. 2B and 4C). Therefore, we next examined the effect of Ac-DEVD-CHO on Bcl-2 phosphorylation. As shown in Fig. 4D, Ac-DEVD-

CHO alone did not induce phosphorylation of Bcl-2, and inhibition of caspase-3 activity by Ac-DEVD-CHO did not affect vinblastine-induced phosphorylation of Bcl-2. Additionally, the expression level of Bax protein was not affected by Ac-DEVD-CHO (Fig. 4D).

Vinblastine-induced G2/M arrest is not affected by Ac-DEVD-CHO Although Ac-DEVD-CHO suppressed vinblastine-induced apoptosis, it was not clear whether Ac-DEVD-CHO suppressed vinblastine-induced G2/M arrest. To address this question, we investigated the effect of Ac-DEVD-CHO on vinblastine-induced G2/M arrest, as determined by flow cytometric analysis of DNA content. As shown in Fig. 5, the percentage of 2C peak (G1 phase) in untreated cells was about 70%, and Ac-DEVD-CHO alone did not affect the distribution of the DNA content in Ms-1 cells. Treatment of Ms-1 cells with 0.1 μg/ml of

vinblastine for 45 h caused a significant increase in both the 4C peak (36%) representing G2/M and the <2C peak (36%) representing apoptosis. On the other hand, in the presence of Ac-DEVD-CHO, vinblastine increased the 4C peak (62.5%), which was accompanied by a decrease in the <2C amount of DNA content (10.5%). Thus, Ac-DEVD-CHO suppressed vinblastine-induced apoptosis, but did not inhibit vinblastine-induced G2/M arrest, thus indicating that caspase-3 was not involved in vinblastine-induced G2/M arrest.

Vinblastine-induced apoptosis is independent of G2/M arrest As shown in Fig. 6A, vinblastine-induced apoptosis seemed to be a consequence of G2/M arrest. For confirmation of this, Ms-1 cells synchronized at late G1 phase by hydroxyurea (HU) were released from the HU block, and then the cells were treated with vinblastine.
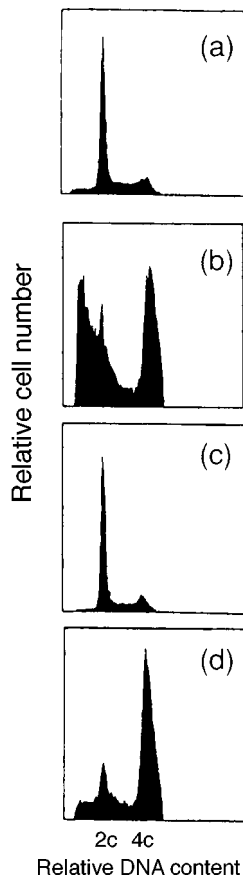

Fig. 5. Effect of Ac-DEVD-CHO on vinblastine-induced G2/M arrest in Ms-1 cells. Ms-1 cells preincubated with 100 μM Ac-DEVD-CHO for 2 h were incubated with or without 0.1 μg/ml of vinblastine. After 45 h, the cells were stained with propidium iodide and their DNA content was evaluated by flow cytometry. a, control; b, vinblastine; c, Ac-DEVD-CHO; d, vinblastine and Ac-DEVD-CHO.
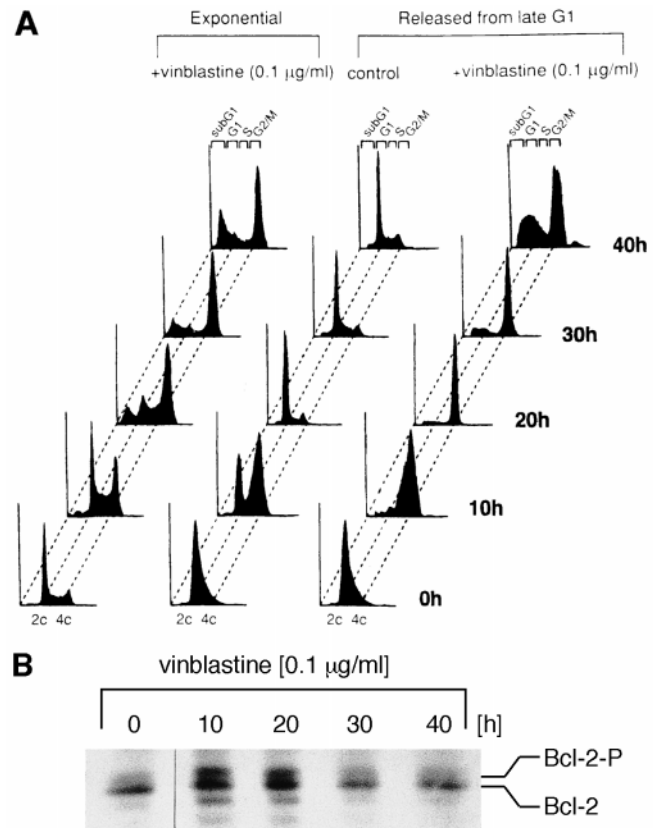

Fig. 6. Induction of apoptosis by vinblastine in synchronized Ms-1 cells. Ms-1 cells released from late G1 phase were treated with 0.1 μg/ml of vinblastine. (A) At the indicated times, the cells were stained with propidium iodide and their DNA content was evaluated by flow cytometry. (B) Samples were prepared at the indicated times, and analyzed by western blotting using anti-Bcl-2 antibody.

Vinblastine-induced G2/M arrest in HU-released cells was observed 10 h earlier than that in asynchronized cells. Nevertheless, DNA fragmentation in cells released from the HU block was first detected at the same time point as found for the asynchronized cells. On the other hand, Bcl-2 phosphorylation was observed at 10 h after treatment with vinblastine in cells released from G1 phase as well as in asynchronized cells (Figs. 2B and 6B). These results suggest that the duration of the G2/M phase was not required for the induction of apoptosis by vinblastine.

DISCUSSION

In the present study, we demonstrated that the phosphorylation of Bcl-2 protein may be involved in vinblastine-induced apoptosis. The phosphorylation of Bcl-2 protein was shown originally in phosphatase inhibitor-treated leukemic cells to result in apoptosis.[23] Furthermore, it has been shown that treatment of human carcinomas with taxol as well as vinblastine can lead to Bcl-2 phosphorylation, and results in cell death, suggesting that phosphorylation of Bcl-2 diminishes its function.[19, 20, 24–26] In contrast, Ito *et al*. reported that Bcl-2 phosphorylation at Ser70 induced by IL-3 or bryostatin-1 is required for full Bcl-2 death suppressor signaling activity, and this phosphorylation might be mediated by protein kinase C.[27] Vinblastine-induced Bcl-2 phosphorylation was not inhibited by protein kinase C inhibitor, calphostine C (data not shown). Therefore, the phosphorylation site in Bcl-2 induced by vinblastine seems to be different from that induced by IL-3 or bryostatin-1. This difference may explain the opposite function of Bcl-2 on apoptosis. We also found that the phosphorylated Bcl-2 induced by vinblastine is incapable of forming heterodimers with Bax protein. Similarly, it was reported that taxol-induced phosphorylation of Bcl-2 is followed by increased intracellular free Bax levels.[25] The Bax homodimer is considered to be a functional form involved in acceleration of cell death.[24, 28] Recently, it was reported that Bax homodimerization is not required for Bax to accelerate chemotherapy-induced cell death.[29] Therefore, monomeric or homodimeric Bax released from phosphorylated Bcl-2 following vinblastine treatment might accelerate apoptosis.

On the other hand, vinblastine-induced apoptosis was suppressed by Ac-DEVD-CHO, an inhibitor of caspase-3, indicating that the activation of caspase-3 is responsible for vinblastine-induced apoptosis. Indeed, the 17-kDa activated caspase-3 fragment was detected following vinblastine treatment in Ms-1 cells. However, Ac-DEVD-CHO did not affect vinblastine-induced Bcl-2 phosphorylation, indicating that Bcl-2 phosphorylation is not regulated by caspase-3 activation. This finding is consistent with previous studies showing that, in Bcl-X$_L$-overexpressing cells, taxol did not induce caspase-3 activation but induced phosphorylation of Bcl-2.[25] It was reported that tetracycline-inducible Bax expression activated caspase-3.[28] Furthermore, it was shown that overexpression of Bcl-2 or Bcl-X$_L$ suppressed taxol-induced caspase-3 activation and apoptosis.[16, 25] Taking these results together with our present findings, it seems likely that vinblastine-induced inactivation of Bcl-2 by the phosphorylation, leading to Bax activation, resulted in caspase-3 activation. On the other hand, the inhibitor of caspase-3 did not affect the vinblastine-induced G2/M arrest, suggesting that vinblastine-induced caspase-3 activation was not involved in the G2/M arrest induced by vinblastine.

It has been considered that vinblastine-induced cell death is initiated by vinblastine-induced G2/M phase cell-cycle arrest and subsequent cumulative cytotoxicity. Bcl-2 responds to damage in microtubules and is phosphorylated at the G2/M phase.[24, 26] Our findings indicate that apoptosis is independent of the duration of the G2/M phase, since apoptosis was induced by vinblastine equally in cycling and HU-released cells. Taken together, the trigger of vinblastine-induced apoptosis might be Bcl-2 phosphorylation in the G2/M phase, which may occur independently of G2/M duration. It is more likely that microtubule damage by vinblastine leads to two independent cellular events, apoptosis and G2/M arrest.

REFERENCES

1) Jordan, M. A., Wendell, K., Gardiner, S., Derry, W. B., Copp, H. and Wilson, L. Mechanism of inhibition of cell proliferation by Vinca alkaloids. *Cancer Res.*, **51**, 2212–2222 (1991).
2) Howard, S. M. H., Theologides, A. and Sheppard, J. R. Comparative effects of vindesine, vinblastine, and vincristine on mitotic arrest and hormonal response of L1210 leu-
kemia cells. *Cancer Res.*, **40**, 2695–2700 (1980).
3) Wendell, K. L., Wilson, L. and Jordan, M. A. Mitotic block in HeLa cells by vinblastine: ultrastructural changes in kinetochore-microtubule attachment and in centrosomes. *J. Cell Sci.*, **104**, 261–272 (1993).
4) Donehower, L. A. and Bradley, A. The tumor suppressor p53. *Biochim. Biophys. Acta*, **1155**, 181–205 (1993).

5) Zambetti, G. P. and Levine, A. J. A comparison of the biological activities of wild-type and mutant p53. *FASEB J.*, **7**, 855–865 (1993).

6) Yuan, J., Shahan, S., Lendoux, S., Ellis, H. M. and Horvitz, H. R. The *C. elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1β-converting enzyme. *Cell*, **75**, 641–652 (1993).

7) Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G. and Earnshaw, W. C. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature*, **371**, 346–347 (1994).

8) Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T. T., Yu, V. L. and Miller, D. K. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature*, **376**, 37–43 (1995).

9) Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S. and Dixit, V. M. Yama/CPP32β, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell*, **81**, 801–809 (1995).

10) Hengartner, M. O. and Horvitz, H. R. Programmed cell death in *Caenorhabditis elegans*. *Curr. Opin. Genet. Dev.*, **4**, 581–586 (1994).

11) Hengartner, M. O. and Horvitz, H. R. *C. elegans* cell-survival gene Ced-9 encodes a functional homolog of the mammalian protooncogene Bcl-2. *Cell*, **76**, 665–676 (1994).

12) Chresta, C. M., Masters, J. R. W. and Hickman, J. A. Hypersensitivity of human testicular tumors to etoposide-induced apoptosis is associated with functional p53 and a high Bax:Bcl-2 ratio. *Cancer Res.*, **56**, 1834–1841 (1996).

13) Hockenbery, D. M., Oltvai, Z. N., Yin, X.-M., Milliman, C. L. and Korsmeyer, S. J. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell*, **75**, 241–251 (1993).

14) Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord, T. and Bredesen, D. E. Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science*, **262**, 1274–1277 (1993).

15) Shimizu, S., Eguchi, Y., Kamiike, W., Matsuda, H. and Tsujimoto, Y. Bcl-2 expression prevents activation of the ICE protease cascade. *Oncogene*, **12**, 2251–2257 (1996).

16) Ibrado, A. M., Huang, Y., Fang, G. and Bhalla, K. Bcl-x$_L$ overexpression inhibits taxol-induced Yama protease activity and apoptosis. *Cell Growth Differ.*, **7**, 1087–1094 (1996).

17) Ibrado, A. M., Huang, Y., Fang, G., Liu, L. and Bhalla, K. Overexpression of Bcl-2 or Bcl-x$_L$ inhibits Ara-C-induced CPP32/Yama protease activity and apoptosis of human acute myelogenous leukemia HL-60 cells. *Cancer Res.*, **56**, 4743–4748 (1996).

18) Guan, R. J., Khatra, B. S. and Cohlberg, J. A. Phosphorylation of bovine neurofilament proteins by protein kinase FA (glycogen synthesis kinase 3). *J. Biol. Chem.*, **266**, 8262–8267 (1991).

19) Haldar, S., Chintapalli, J. and Croce, C. M. Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. *Cancer Res.*, **56**, 1253–1255 (1996).

20) Blagosklonny, M., Schulte, T., Nguyen, P., Trepel, J. and Neckers, L. M. Taxol-induced apoptosis and phosphorylation of Bcl-2 protein involves c-Raf-1 and represents a novel c-Raf-1 signal transduction pathway. *Cancer Res.*, **56**, 1851–1854 (1996).

21) Guan, R. J., Moss, S. F., Arber, N., Krajewski, S., Reed, J. C. and Holt, P. R. 30 kDa phosphorylated form of Bcl-2 protein in human colon. *Oncogene*, **12**, 2605–2609 (1996).

22) Erhardt, P. and Cooper, G. M. Activation of the CPP32 apoptotic protease by distinct signaling pathways with differential sensitivity to Bcl-x$_L$. *J. Biol. Chem.*, **271**, 17601–17604 (1996).

23) Haldar, S., Jena, N. and Croce, C. M. Inactivation of Bcl-2 by phosphorylation. *Proc. Natl. Acad. Sci. USA*, **92**, 4507–4511 (1995).

24) Haldar, S. Basu, A. and Croce, C. M. Bcl2 is the guardian of microtubule integrity. *Cancer Res.*, **57**, 229–233 (1997).

25) Ibrado, A. M., Liu, L. and Bhalla, K. Bcl-X$_L$ overexpression inhibits progression of molecular events leading to paclitaxel-induced apoptosis of human acute myeloid leukemia HL-60 cells. *Cancer Res.*, **57**, 1109–1115 (1997).

26) Blagosklonny, M. V., Giannakakou, P., El-Deiry, W. S., Kingston, D. G. I., Higgs, P. I., Neckers, L. and Fojo, T. Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death. *Cancer Res.*, **57**, 130–135 (1997).

27) Ito, T., Deng, X., Carr, B. and May, W. S. Bcl-2 phosphorylation required for anti-apoptosis function. *J. Biol. Chem.*, **272**, 11671–11673 (1997).

28) Xiang, J., Chao, D. T. and Korsmeyer, S. J. Bax-induced cell death may not require interleukin 1β-converting enzyme-like proteases. *Proc. Natl. Acad. Sci. USA*, **93**, 14559–14563 (1996).

29) Simonian, P. L., Grillot, D. A. M., Andrews, D. W., Leber, B. and Nunez, G. Bax homodimerization is not required for Bax to accelerate chemotherapy-induced cell death. *J. Biol. Chem.*, **271**, 32073–32077 (1996).