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Research Articles

Calphostin C synergistically induces apoptosis with VP-16 in lymphoma cells which express abundant phosphorylated Bcl-2 protein

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Abstract. A newly established human lymphoma cell line (OZ) has the t(14;18)(q32;q21) translocation and expresses large amounts of Bcl-2 compared to CCRF-CEM cells. VP-16 (40 μg/mL), a promising agent against lymphoma, caused DNA fragmentation (26.9% of total DNA) typical for apoptosis at 6 h in CCRF-CEM cells, but no significant changes in OZ cells until 24 h after the addition of VP-16. However, coincubation with calphostin C (0.2 μg/mL), a protein kinase C (PKC) inhibitor, induced DNA fragmentation in VP-16-treated OZ cells (13.5% of total DNA) at 6 h after the treatment. Simultaneous immunoblot analysis revealed that this induction of apoptosis coincided with the downregulation of serine-phosphorylated Bcl-2 (13% of control cells). By contrast, apoptosis induced by VP-16 in CCRF-CEM cells was attenuated by the addition of 0.5 μM phorbol 12-myristate 13-acetate, a potent PKC stimulator. These observations suggest that Bcl-2 function is partly regulated by phosphorylation/dephosphorylation mechanisms of the PKC system, and that phosphorylated Bcl-2 in lymphoma cells may play a role in the prevention of apoptosis.

Key words. Apoptosis; Bcl-2; protein kinase C; follicular lymphoma; phosphorylation.

Recent studies have implicated activation of certain oncogenes in the pathogenesis of lymphoma, frequently in association with specific chromosomal translocations [1]. The translocation t(14;18)(q32;q21) is a common finding in follicular lymphoma [2], and cloning of the breakpoint of 18q21 has resulted in the identification of the *bcl-2* oncogene [3, 4]. In this translocation, the *bcl-2* gene is juxtaposed to the immunoglobulin heavy chain (IgH) genes on 14q32, and *bcl-2* is overexpressed by the strong promotor of IgH [5]. Because the chromosomal breakpoint falls outside the translated portions of the *bcl-2* gene, the protein product is identical to normal Bcl-2, and the high expression of Bcl-2 has been postulated to play an important role in lymphoma pathogenesis [6]. Unlike other oncogenes, *bcl-2* has shown no demonstrable influence on cell proliferation; instead *bcl-2* extends cell survival by blocking apoptosis [7]. Since several chemotherapeutic agents have been demonstrated to cause apoptosis in tumour cells [8], it is important to clarify the actions of the overexpressed Bcl-2 to develop novel therapies against follicular lymphoma.

Recently, we have established a novel human pre-B cell line termed OZ from the bone marrow of a patient with malignant lymphoma. OZ cells have several karyotypic abnormalities including t(14;18)(q32;q21), and they overexpress Bcl-2 as expected. Interestingly, immunoblot analysis has demonstrated that OZ cells contain large amounts of serine-phosphorylated Bcl-2 proteins. To determine the significance of this phosphorylation, we

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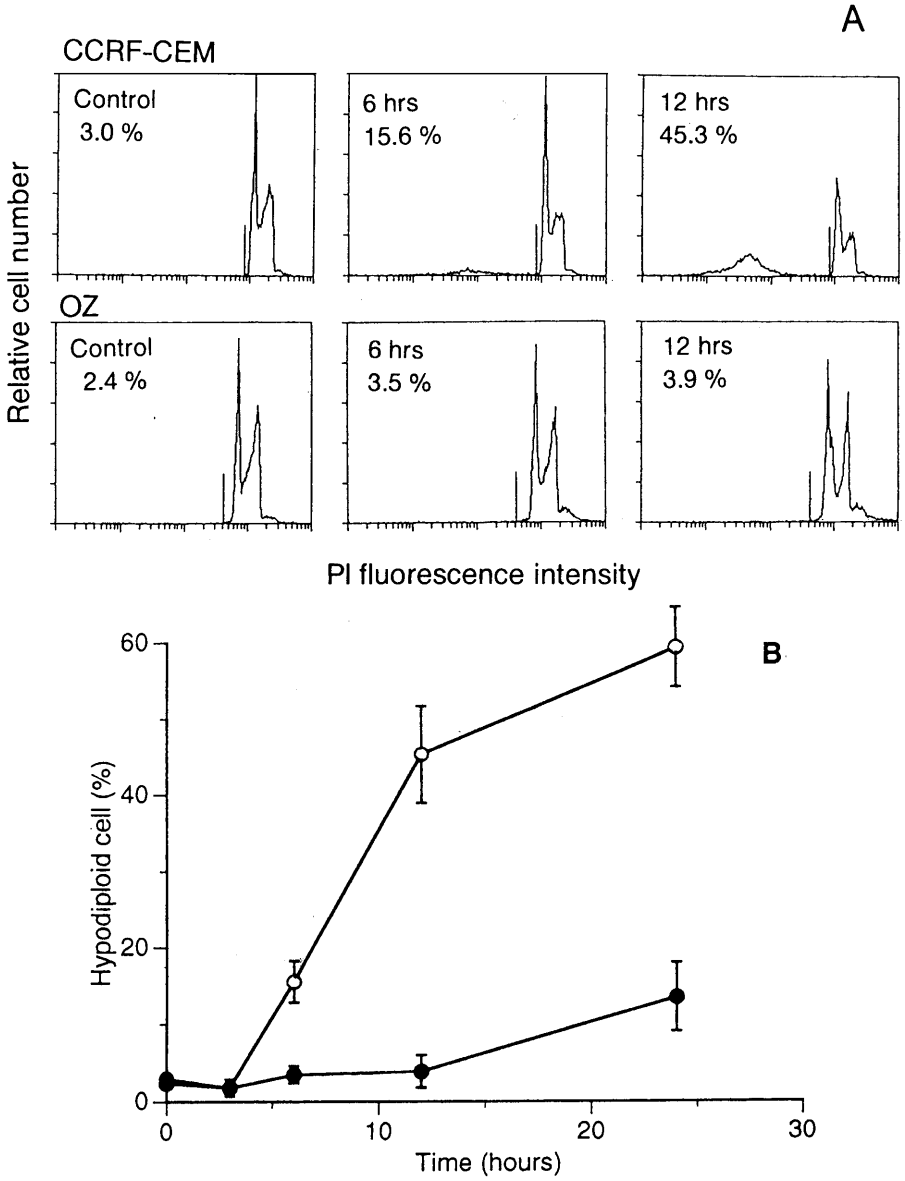

Figure 1. Changes in cell cycle distribution and apoptosis following VP-16. CCRF-CEM and OZ cells were cultured with VP-16 (40 μg/ml) for the indicated periods, then permeabilized, incubated with propidium iodide (PI), and analysed by flow cytometry. (*A*) Nuclei of apoptotic cells display a PI staining intensity which is lower than that of nuclei from untreated cells with diploid content. (*B*) Time-dependent increase in hypodiploid/apoptotic cells in OZ (closed circle) and CCRF-CEM cells (open circle). The values indicate the mean ± SD of triplicate cultures.

examined whether activation or inhibition of protein kinase C (PKC), which has strong serine kinase activity, affects Bcl-2 function in preventing apoptosis and extending cell survival.

Materials and methods

Cell culture. OZ cells have been in continuous culture since March 1993 in our laboratory and express CD10 and CD19 but not surface immunoglobulin. CCRF-CEM cells derived from an acute lymphoblastic leukemia with a thymic T cell phenotype were provided by the Japanese Cancer Research Resources Bank (JCRB, Tokyo, Japan). Both cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin 100 U/mL and streptomycin 100 μg/mL, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Reagents. VP-16 was the gift of Nippon Kayaku Co. Ltd. (Tokyo, Japan). Calphostin C and phorbol 12-myristate 13-acetate (TPA) were purchased from Fu-

nakoshi Co. Ltd. (Tokyo, Japan). Monoclonal anti-human Bcl-2 antibody (clone 124) was obtained from DAKO (Carpinteria, CA). Anti-human anti-phosphoserine (clone PSR-45) and anti-phosphothreonine (clone PTR-8) antibodies were purchased from BioMakor (Rehovot, Israel). Anti-mouse IgG was obtained from Wako Pure Chemical (Osaka, Japan). ^{125}I-Protein A was obtained from ICN (Tokyo, Japan).

Assessment of apoptosis. Flow cytometry was used to identify hypodiploid/apoptotic cells and to measure the percentage of hypodiploid cells following propidium iodide (PI) staining in hypotonic buffer, as previously described [9]. Briefly, cell pellets were suspended in 1 ml hypotonic fluorochrome solution (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X; Sigma), and cells were analysed in a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) with Cell-Fit software. Hypodiploid/apoptotic cells appeared as cells with DNA content less than G1. Chromatin cleavage was determined as reported previously [10]. The percentage of chromatin cleavage was defined as the ratio of the concentration of supernatant DNA to total DNA determined by the spectrophotometer. The statistical analysis was performed using Student's t-test.

Western blot analysis. Western blot analysis was performed as described previously [11]. Briefly, 5×10^6 cells were lysed and denatured in sample buffer and separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a nitrocellulose membrane with Transblot SD (Bio-Rad, Tokyo, Japan). The membrane blots were rinsed with TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween 20) and blocked with skim milk. The blots were incubated with mouse anti-human Bcl-2 monoclonal antibody followed by rabbit anti-mouse IgG. Bcl-2 protein then was detected by ^{125}I-protein A, and quantitative analysis was done with the Bioimaging analyser (Fuji Co. Ltd., Tokyo, Japan). Phosphorylated Bcl-2 protein was measured by Western blot analysis using anti-human anti-phosphoserine or phosphothreonine monoclonal antibodies following immuno- precipitation by anti-human Bcl-2 monoclonal antibody. Blot detection was as described above.

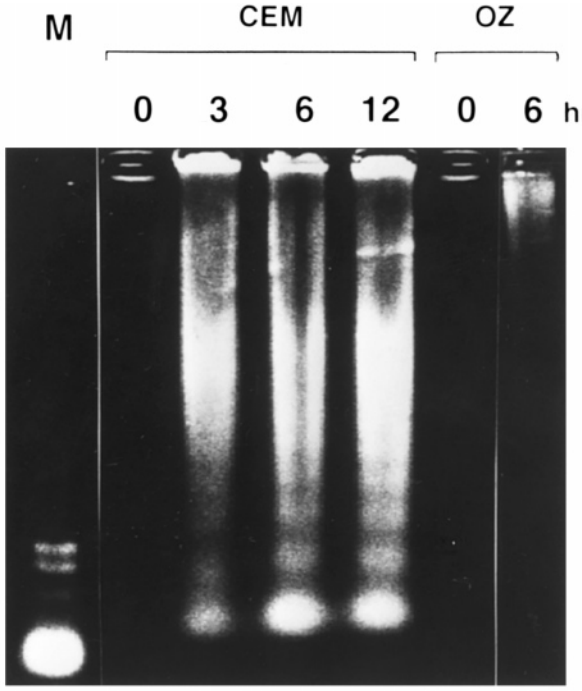

Figure 2. Oligosomal DNA fragmentation induced by VP-16. OZ and CCRF-CEM cells were cultured with VP-16 (40 μg/ml), and DNA fragmentation was evaluated at various time points. M is ΔX174, *Hae*III-digested marker. Each lane corresponds to the DNA fragments from 2×10^6 cells.

Results

Effects of VP-16 on apoptosis induction in CCRF-CEM and OZ cells. Figure 1A shows the progression of cell cycle distribution of CCRF-CEM and OZ cells following treatment with 40 μg/ml VP-16. In CCRF-CEM cells, progressive cell cycle arrest and an increase in the percentage of hypodiploid cells were observed; hypodiploid cells increased to 15.6% at 6 h and 45.3% at 12 h following the addition of VP-16. By contrast, VP-16 had no effect on OZ cells until 24 h when the percentage of hypodiploid cells increased to 13.6% (fig. 1B). This difference in susceptibility to apoptosis induction was significant ($p < 0.01$). In addition to flow cytometric analysis, we simultaneously carried out DNA laddering analysis. As shown in figure 2, 3 h after the addition of VP-16, nucleosomal ladders, characteristics

Table 1. Apoptosis induction in OZ and CCRF-CEM cells.

Treatment	% of fragmented DNA	% of hypodiploid cells
OZ cells		
Control	3.6 ± 1.6	2.4 ± 0.4
Calphostin C (0.2 μg/ml)	2.4 ± 2.1	2.8 ± 1.1
VP-16 (40 μg/ml)	7.1 ± 1.3*	3.5 ± 1.1
VP-16 + Calphostin C	13.5 ± 1.1*,**	9.2 ± 3.2*,**
CCRF-CEM cells		
Control	2.0 ± 2.2	3.0 ± 0.3
Calphostin C (0.2 μg/ml)	3.3 ± 1.2	4.2 ± 0.7
TPA (0.5 μM)	5.6 ± 1.0	5.0 ± 1.2
VP-16 (40 μg/ml)	26.9 ± 0.9*	15.6 ± 1.7*
VP-16 + Calphostin C	28.3 ± 1.2*	20.4 ± 1.5*,**
VP-16 + TPA	11.7 ± 1.5*,**	10.1 ± 1.3*,**

Cells were harvested 6 h following treatment with the agents shown. The values are the mean ± SD of triplicate cultures. Asterisks indicate a significant difference ($p < 0.05$) from *control cells or **VP-16-treated cells.

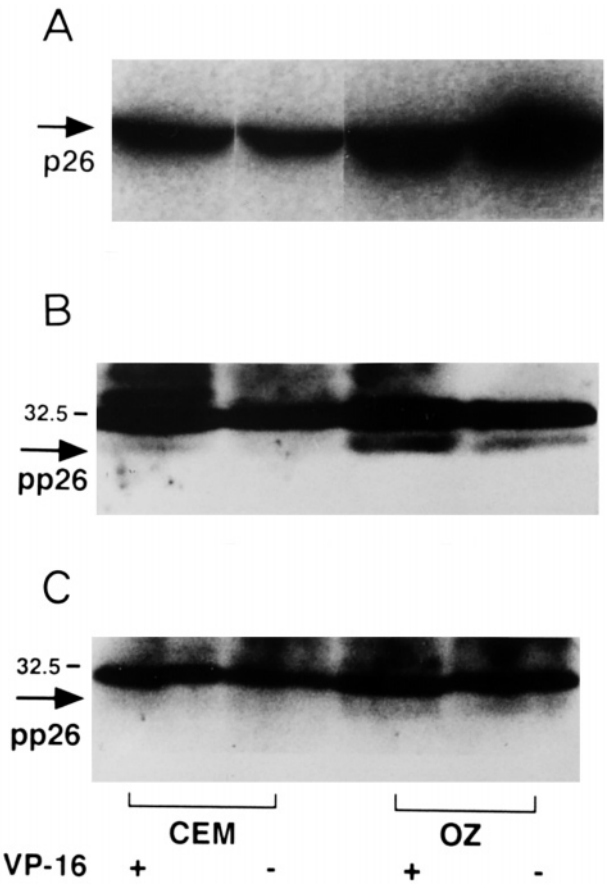

Figure 3. Expression and phosphorylation status of Bcl-2 in CCRF-CEM and OZ cells in the absence or presence of VP-16 (40 μg/ml, 6 h). (*A*) 5×10^6 cells were lysed and detected using anti-human Bcl-2 monoclonal antibody. The arrow indicates the 26 kD Bcl-2 protein. Expression of serine (*B*) and threonine (*C*)-phosphorylated Bcl-2. Immunodetection was performed using monoclonal antibody specific for serine- or threonine-phosphorylated protein following immunoprecipitation by anti-human Bcl-2 monoclonal antibody. The arrows indicate phosphorylated Bcl-2 (pp26) protein and thick bands at 32.5 kD correspond to immunoglobulin light chains.

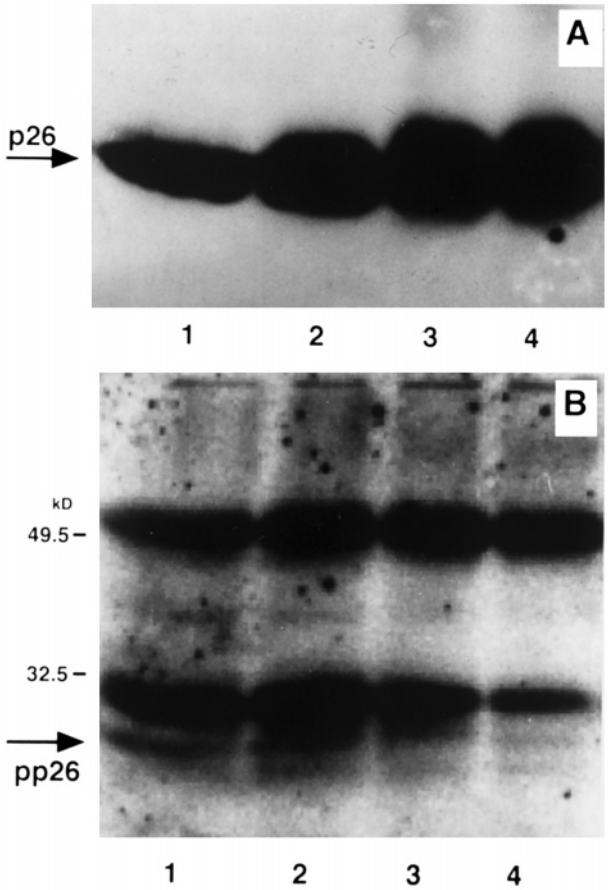

Figure 4. Western blot analysis of total and phosphorylated Bcl-2 in OZ cells following VP-16 (40 μg/ml) and/or calphostin C (0.2 μg/ml) treatment for 6 h. Expression of (*A*) total Bcl-2 (p26 Bcl-2) and (*B*) serine-phosphorylated Bcl-2 (pp26 Bcl-2): lane 1, control; 2, VP-16 alone; 3, calphostin C alone; 4, VP-16 plus calphostin C. Phosphorylated Bcl-2 was detected using monoclonal antibody specific for serine-phosphorylated protein following immunoprecipitation by anti-human Bcl-2 monoclonal antibody. The thick bands at 49.5 and 32.5 kD correspond to immunoglobulin heavy and light chains, respectively.

for apoptosis, could be demonstrated. Spectrophotometrical analysis revealed that 26.9% of the total DNA had been degraded to oligosomal fragments by 6 h after the treatment. Morphologically, marked nuclear condensation and fragmentation were observed in these cells (data not shown). In OZ cells, by contrast, typical nucleosomal fragmentation in apoptosis was not observed, although degraded DNA accounted for 7.1% of total DNA (table 1, fig. 2). These findings suggest that OZ cells are less susceptible to apoptosis induction by VP-16 than CCRF-CEM cells.

Upregulation of phosphorylated Bcl-2 during the early phase of apoptosis induced by VP-16. To examine the possible involvement of Bcl-2 in the apoptosis induced by VP-16, the expression and phosphorylated status of Bcl-2 were analysed. As shown in figure 3A, OZ cells expressed more abundant Bcl-2 than did CCRF-CEM cells. However, no significant changes in Bcl-2 expression were found at 6 h following addition of VP-16 in either cell line. We simultaneously compared the phosphorylation status of Bcl-2 in these cells. Immunoblot analysis using a monoclonal antibody specific for serine-phosphorylated proteins following immunoprecipitation by anti-Bcl-2 antibody, revealed that OZ cells contained twice as much phosphorylated Bcl-2 than CCRF-CEM cells. Interestingly, these phosphorylated Bcl-2 were upregulated at 6 h following the addition of VP-16 in both cell lines (fig. 3B). By contrast, threonine-phosphorylated proteins were not found in either cell line (fig. 3C).

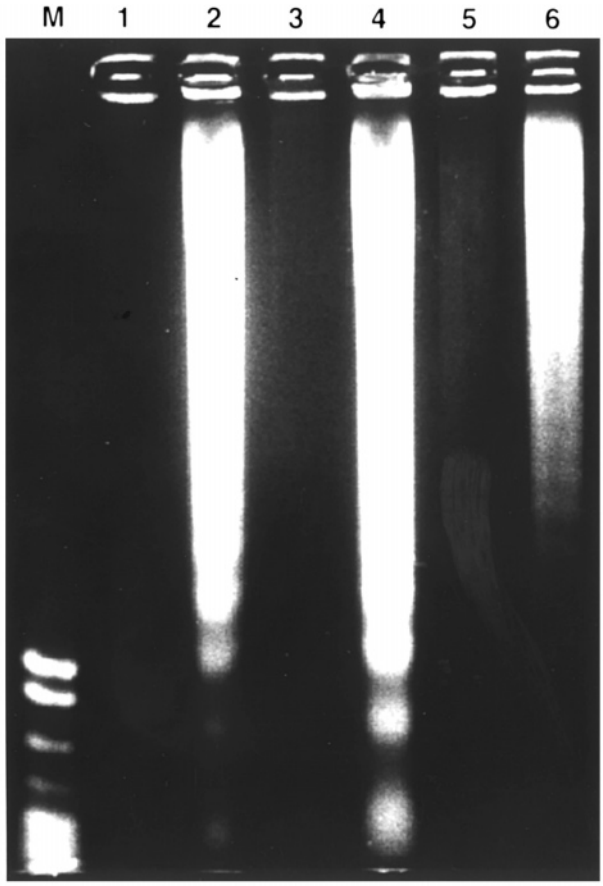

Figure 5. Oligosomal DNA fragmentation in CCRF-CEM cells induced by treatment with the various agents for 6 h: lane 1, control; 2, VP-16 (40 μg/ml); 3, calphostin C (0.2 μg/ml); 4, VP-16 plus calphostin C; 5, TPA (0.5 μM); 6, VP-16 plus TPA; M, ΔX174, *Hae* III-digested marker.

Effect of calphostin C on apoptosis induction in VP-16-treated OZ cells. To define the significance of Bcl-2 phosphorylation, we next examined the effect of calphostin C, a potent inhibitor of PKC [12], on the induction of apoptosis in VP-16-treated OZ cells. Flow cytometric and DNA laddering analysis revealed that 0.2 μg/ml calphostin C by itself had no effect on cell cycle distribution or the number of apoptotic cells. However, in combination with VP-16, calphostin C caused apoptotic cells to increase to 13.5% by DNA laddering and 9.2% by flow cytometry by 6 h after the treatment (table 1). Simultaneous immunoblot analysis revealed that the addition of calphostin C did not influence the expression of total Bcl-2, but reduced the level of serine-phosphorylated Bcl-2 to 13% of control cells densitometrically (fig. 4). These observations suggest that the level of phosphorylated Bcl-2, but not total Bcl-2, has a role in prevention of apoptosis.

Effects of TPA and calphostin C on apoptosis induction in VP-16-treated CCRF-CEM cells. As shown in figure 5, treatment with VP-16 (40 μg/ml) resulted in oligosomal DNA fragmentation to 26.9% of total DNA in CCRF-CEM cells (lane 2). However, when combined with TPA the apoptotic effect of VP-16 was attenuated; only 11.7% of DNA was fragmented (lane 6). Flow cytometric analysis confirmed the protective effect of TPA; the percentage of apoptotic cells decreased from 15.6% when treated with VP-16 alone to 10.1% after VP-16 and TPA. By contrast, treatment with calphostin C enhanced the apoptotic effect of VP-16 in CCRF-CEM cells. Fragmented DNA increased to 28.3% (lane 4) and apoptotic cells examined by flow cytometry increased to 20.4% (table 1).

Discussion

Recent observations suggest that protein modification such as phosphorylation/dephosphorylation, rather than de novo protein synthesis, plays an important role in the apoptotic process of human cells [13, 14]. It is possible that Bcl-2 function is regulated by phosphorylation. Indeed, Alnemri et al. reported that Bcl-2 was post-translationally phosphorylated in SF-9 insect cells [15]. More recently, Bcl-2 in colon cancer cells has been demonstrated to be phosphorylated on serine residues [16]. As for the functional significance of this phosphorylation, May et al. have reported that serine phosphorylation of Bcl-2 by bryostatin, a potent activator of PKC, suppressed apoptosis in a murine IL-3-dependent cell line [17]. We have now examined the significance of phosphorylation of Bcl-2 in follicular lymphoma. The newly established OZ cell line is useful because of its overexpression of Bcl-2.

We first examined the effect of VP-16 on cell cycle distribution and survival of OZ and CCRF-CEM cells. VP-16 (etoposide) is an epipodophyllotoxin and a promising agent against lymphoma. VP-16 inhibits topoisomerase II activity by stabilization of the cleavable complex between the enzyme and DNA and formation of protein-bound double-stranded DNA breaks [18], leading to growth arrest and subsequent apoptosis in both normal and malignant cells [8]. Several mechanisms involved in this process have been reported that VP-16 activates poly-ADP-ribosylation [19], ICE (interleukin-1β converting enzyme)-like protease [20], or which induces aberrant DNA recombination [21]; however, the precise mechanisms have not been thoroughly understood. OZ cells were found to be less sensitive than CCRF-CEM cells to apoptotic induction by VP-16. When DNA integrity was analysed by agarose gel electrophoresis, a nucleosomal ladder was evident in CCRF-CEM cells 3 h after the addition of VP-16, and this ladder increased in intensity over 12 h. By contrast, oligonucleosomal DNA laddering was not observed in OZ cells. To clarify this differential susceptibility to apoptosis induction, we examined the phosphorylation

status of Bcl-2 in both cell lines. Immunoblot analysis revealed that Bcl-2 in OZ cells contained more abundant serine-phosphorylated protein than CCRF-CEM cells. It is worth noting that treatment with VP-16 transiently upregulated the level of phosphorylated Bcl-2 in both cell lines at 6 h following the addition of VP-16, when 15.6% of CCRF-CEM cells were hypodiploid. This upregulation was, however, a transient phenomenon and not observed at 12 h (data not shown). Considering its function, this hyperphosphorylation of Bcl-2 might be induced as a counteractive mechanism following exposure to injurious stimuli.

In order to prove this thesis, we next examined the effects of PKC on apoptosis induction by VP-16. PKC has strong serine kinase activity and has been shown to be involved in intracellular signal transduction [22]. In addition, the primary sequence of Bcl-2 contains several PKC consensus serine phosphorylation sites [23], making it a suitable substrate for activated PKC. Treatment with calphostin C, a specific inhibitor of PKC [12], overcame the refractoriness to apoptosis induction by VP-16 in OZ cells. Simultaneous immunoblot analysis revealed that significant amounts of Bcl-2 were still present in OZ cells at 6 h following the addition of VP-16, but its phosphorylated form was downregulated by coincubation with calphostin C at a time when 13.5% of OZ cells showed DNA laddering. These results suggest that phosphorylated Bcl-2 might play a role in preventing apoptosis.

This hypothesis was also supported by the observation that the effects of VP-16 in CCRF-CEM cells were partially blocked by TPA, a potent stimulator of PKC. This attenuating effect of TPA on induction of apoptosis has been demonstrated previously in other cell systems [24–26]. However, there are conflicting observations on the protective effects of TPA against apoptosis. TPA in conjunction with Ca^{2+} ionophore has been reported to induce apoptosis in T-cell hybridoma cells [27]; similarly, an inhibitor of PKC was shown to prevent the formation of apoptotic bodies [28]. Additionally, an apoptosis-promoting effect was observed with okadaic acid [29–31], which causes an increase in phosphorylation of certain proteins not by activating PKC but by inhibiting protein phosphatase. These contrasting effects of phosphorylation may occur with different agents, or in conditions where concentrations and exposure times differ. Since TPA initially activates but then later inhibits PKC activity [32], it is important to consider divergent pharmacodynamic effects of phorbol esters.

Finally, the functional role of serine phosphorylation of Bcl-2 is not yet clear. May et al. have postulated that serine-phosphorylation might increase the stability of the Bcl-2 protein; however, they found no significant difference in the half-life of Bcl-2 of native and hyperphosphorylated forms [16]. The phosphorylation state of Bcl-2 might promote functional interaction between Bcl-2 and other molecules affecting apoptosis, such as Bax [33], Bcl-X [34] and BAG-1 [35].

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Note. OZ cell line is freely distributed to scientists. Requests should be addressed directly to the corresponding author.

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