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Modulation of the expression of Bcl-2 and related proteins in human leukemia cells by protein kinase C activators: relationship to effects on 1-[β-D-arabinofuranosyl]cytosine-induced apoptosis

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Abstract

We have previously reported that pretreatment of HL-60 human promyelocytic leukemia cells with the non-tumor-promoting protein kinase C (PKC) activator bryostatin 1 potentiates induction of apoptosis by the antimetabolite 1-[β-D-arabinofuranosyl]cytosine (ara-C) (Biochem Pharmacol 47:839,1994). To determine whether this phenomenon results from altered expression of Bcl-2 or related proteins, Northern and Western analysis was employed to assess the effects of bryostatin 1 and other PKC activators on steady-state levels of Bcl-2, Bax, Bcl-x, and Mcl-1 mRNA and protein. Pretreatment of cells for 24 h with 10 nM bryostatin 1, or, to a lesser extent, the stage-1 tumor-promoter phorbol dibutyrate (PDB) significantly potentiated apoptosis induced by ara-C (100 μM; 6 h); in contrast, equivalent exposure to the stage-2 tumor promoter, mezerein (MZN), which, unlike bryostatin 1, is a potent inducer of differentiation in this cell line, failed to modify ara-C-related cell death. Neither bryostatin 1 nor PDB altered expression of *bcl-2*/Bcl-2 over this time frame. In contrast, MZN down-regulated *bcl-2* mRNA levels, but this effect was not accompanied by altered expression of Bcl-2 protein. None of the PKC activators modified expression of Bax or Bcl-x_L mRNA or protein; levels of Bcl-x_S were undetectable in both treated and untreated cells. However, expression of Mcl-1 mRNA and protein increased modestly after treatment with either bryostatin 1 or PDB, and to a greater extent following exposure to MZN. Combined treatment of cells with bryostatin 1 and MZN resulted in undiminished potentiation of ara-C-mediated apoptosis and by antagonism of cellular maturation. These effects were accompanied by unaltered expression of Bcl-2, Bax, and Bcl-x_L, and by a further increase in Mcl-1 protein levels. When cells were co-incubated with bryostatin 1 and calcium ionophore (A23187), an identical pattern of expression of Bcl-2 family members was observed, despite the loss of bryostatin 1's capacity to potentiate apoptosis, and the restoration of its ability to induce differentiation. Finally, treatment of cells with bryostatin 1 ± ara-C (but not ara-C alone) resulted in a diffuse broadening of the Bcl-2 protein band, whereas exposure of cells to taxol (250 nM, 6 h) led to the appearance of a distinct Bcl-2 species with reduced mobility, phenomena compatible with protein phosphorylation. Together, these findings indicate that the ability of bryostatin 1 to facilitate drug-induced apoptosis in human myeloid leukemia cells involves factors other than quantitative changes in the expression of Bcl-2 family members, and raise the possibility that qualitative alterations in the Bcl-2 protein, such as phosphorylation status, may contribute to this capacity. They also suggest that increased expression of Mcl-1 occurs early in the pre-commitment stage of myeloid cell differentiation, and that this event does not protect cells from drug-induced apoptosis.

Keywords: apoptosis, *bcl-2*, ara-C, bryostatin 1, protein kinase C

Abbreviations: PKC, protein kinase C; ara-C, 1-[β-D-arabinofuranosyl]cytosine; PDB, phorbol dibutyrate; MZN, mezerein; PMA, 12-phorbol 13-myristate acetate; TNF, tumor necrosis factor; DMSO, dimethylsulfoxide; TPA, tumor promoting agent

Introduction

The proto-oncogene *bcl-2* encodes a 26-kDa protein (Bcl-2) that protects cells from induction of apoptosis by a wide variety of stimuli, including growth factor deprivation, heat shock, and numerous chemotherapeutic drugs, among many others (Reed, 1994). Since its initial discovery and characterization, other homologous proteins have been identified which either promote or oppose apoptotic events. For example, the Bcl-2 homolog Bax forms dimers which induce apoptosis, an action that is antagonized by Bcl-2/Bax heterodimerization (Oltavi *et al*, 1993). Another homolog, Bcl-x, gives rise through an alternative splicing mechanism to a short form (Bcl-x_S) which promotes apoptosis, and a long form (Bcl-x_L), which exerts the opposite effect (Boise *et al*, 1993). Other more recently described family members include Mcl-1 and A1, whose function may be similar to that of Bcl-2 (Kozopas *et al*,

1993; Lin *et al*, 1996), as well as Bad, which facilitates cell death (Yang *et al*, 1995). It has been proposed that the susceptibility of cells to apoptosis depends upon the relative concentrations of pro- and anti-apoptotic genes (Oltavi and Korsmeyer, 1994). Furthermore, overexpression of Bcl-2 by leukemic cells confers resistance to a broad range of antineoplastic drugs (Miyashita and Reed, 1993). The potential clinical significance of this phenomenon in hematopoietic neoplasms has been underscored by evidence that increased expression of Bcl-2 is associated with a poor response to chemotherapy in patients with Hodgkin's disease (Yunis *et al*, 1989) and in AML (Campos *et al*, 1993).

Expression of Bcl-2 in hematopoietic cells is linked to differentiation state. Levels are highest in primitive hematopoietic progenitors, and decline with cellular maturation (Delia *et al*, 1992). In malignant hematopoietic cells, dysregulated expression of Bcl-2 does not prevent maturation in response to all-*trans* retinoic acid or 12-phorbol 13-myristate acetate (PMA), although it does delay the onset of apoptosis following induction of terminal differentiation (Naumovski and Cleary, 1994; Park *et al*, 1994). Given recent evidence that tumor necrosis factor (TNF) and ceramide reduce *bcl-2* mRNA levels in human leukemia cells (Chen *et al*, 1995), it is possible that modulation of Bcl-2 expression by such compounds might increase the susceptibility of neoplastic cells to drug-induced and other forms of apoptosis.

We have previously reported that exposure of HL-60 human leukemia cells to the protein kinase C activator, bryostatin 1, increases their susceptibility to apoptosis induced by the antimetabolite 1-[β-D-arabinofuranosyl]cytosine (ara-C) (Grant *et al*, 1992). Bryostatin 1 variably induces differentiation in HL-60 cells (Kraft *et al*, 1989) but is ineffective in the HL-60 cell line used in these studies (Jarvis *et al*, 1994a). In contrast, the stage-2 tumor promoter mezerein (MZN) is a potent inducer of HL-60 cell differentiation but, unlike bryostatin 1, is unable to potentiate ara-C-mediated apoptosis (*ibid*). In view of evidence linking retinoic acid-induced changes in Bcl-2 expression to altered drug sensitivity (Hu *et al*, 1996), it seemed plausible that the modulatory effects of bryostatin 1 and other PKC activators on ara-C-induced apoptosis might be related to specific perturbations in expression of members of this gene family. Currently, little is known about the early effects of bryostatin 1 and other PKC activators on the expression of Bcl-2 and its homologs in human myeloid leukemia cells, particularly in relation to drug sensitivity. The present study was prompted by a desire to compare the effects of bryostatin 1, MZN, and the stage-1 tumor promoter phorbol dibutyrate (PDB) on expression of Bcl-2, Bax, Bcl-x, and Mcl-1 in HL-60 cells at both the message and protein levels, and to determine whether such changes, presuming they occur, may be correlated with alterations in the susceptibility of these cells to ara-C-induced apoptosis.

Results

The effects of preincubating cells for 24 h with 10 nM bryostatin 1, PDB, or MZN on apoptosis induced by ara-C (10 μM, 6 h) are shown in Figure 1A. Whereas exposure of cells to ara-C alone induced apoptosis in approximately 18% of cells, pre-incubation with bryostatin 1 increased this value by over 100%. An identical concentration of PDB was less effective in this regard, although a significant increase in apoptosis was still noted compared to cells treated with ara-C alone (e.g., $\sim$68%; $P < 0.02$). In contrast, 10 nM MZN was completely ineffective in potentiating ara-C-induced apoptosis. Parallel results were obtained when DNA fragmentation

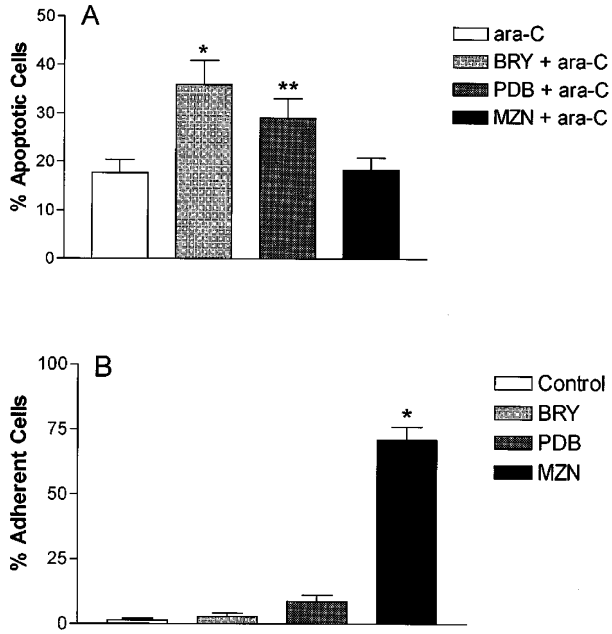

Figure 1 HL-60 cells were incubated with 10 nM bryostatin 1 (BRY), PDB or MZN for 24 h, followed by a 6-h exposure to 10 μM ara-C (**A**). The percentage of apoptotic cells in Wright Giemsa-stained preparations was determined by scoring $\geqslant$500 cells/condition. Alternatively, following a 24-h incubation with the same agents, the percentage of adherent cells was determined as described in the text (**B**). Values represent the means for triplicate determinations $\pm$S.D. Significance of differences compared to ara-C alone (**A**) or controls: *=$P \leqslant 0.02$; **=$P \leqslant 0.05$.

Table 1

Condition	DNA fragmentation (ng DNA/10^6 cells)
1. Control	258$\pm$41
2. ara-C (10 μM)	487$\pm$55
3. BRY (10 nM)+ara-C	1026$\pm$129*
4. PDB (10 nM)+ara-C	789$\pm$87**
5. MZN (10 nM)+ara-C	564$\pm$69
6. BRY+MZN+ara-C	985$\pm$102*
7. A23+BRY	299$\pm$43
8. A23+BRY+ara-C	578$\pm$76

Logarithmically growing cells were exposed to the designated concentration of BRY, PDBu (PDB), mezerein (MZN), or A23187 (A23; 250 nM) for 24 h followed by a 6 h incubation with 10 μM ara-C. At the end of this period, cells were pelleted, lysed, and low molecular weight DNA fragments quantified as described in Materials and Methods. Cells exposed to BRY, PDB, MZN, or A23 alone for 24 h displayed fragmentation equivalent to that of untreated cells (not shown). Values represent the means for three separate experiments performed in triplicate$\pm$S.D. P values (relative to ara-C alone): *=$\leqslant$0.01; **=$\leqslant$0.05.

was monitored (Table 1). As previously reported (Jarvis *et al*, 1994b), the ability of these agents to potentiate ara-C-mediated apoptosis was inversely correlated with their capacity to induce cellular maturation, reflected by the percentage of cells exhibiting plastic adherence (Figure 1B).

In view of previous reports that exposure of leukemic cells to agents such as ceramide and TNF reduced expression of *bcl-2* message (Chen *et al*, 1995), Northern analysis was performed on RNA extracted from cells following a 24 h exposure to each of these agents (Figure 2A). No alterations in *bcl-2* message were observed in cells exposed to bryostatin 1 or PDB. In contrast, preincubation with MZN, which is an effective inducer of maturation in this subline, resulted in a clear reduction in *bcl-2* mRNA levels. However, when Western analysis was performed, none of the agents induced changes in expression of Bcl-2 protein (Figure 2B), consistent with its long reported half-life (Kitada *et al*, 1994). For example, cells pretreated with bryostatin 1 exhibited Bcl-2 levels equal to $107 \pm 13\%$ of controls (N=3; $P \geqslant 0.05$). Thus, the ability of bryostatin 1 (and, to a lesser extent, PDB) to potentiate ara-C-mediated apoptosis in these cells could not be attributed to down-regulation of Bcl-2 protein. Furthermore, these findings indicate that reductions in *bcl-2* mRNA induced by an effective differentiating agent (MZN) are not accompanied by a parallel decrease in protein expression at the 24-h time interval in which sensitivity to ara-C was measured.

Despite its inability to down-regulate Bcl-2 expression, it remained possible that potentiation of apoptosis by bryotstatin 1 might reflect decreases in the Bcl-2/Bax ratio. To address this possibility, expression of *bax* mRNA and protein was monitored in cells following an identical exposure to each of the PKC activators. It can be seen that *bax* mRNA and protein levels remained essentially unchanged following treatment with all three of the indicated agents (Figure 3A and 3B), a finding that was confirmed quantitatively. Consequently, the ability of bryostatin 1 (and PDB) to augment ara-C-induced apoptosis could not be attributed to decreases in the Bcl-2/Bax ratio. In view of evidence that increased expression of Bcl-x_L protects cells from drug-induced apoptosis (Minn *et al*, 1995), parallel studies were performed examining Bcl-x_L and Bcl-x_S levels (Figure 4). As observed in the case of Bax, none of the agents tested significantly altered expression of the anti-apoptotic Bcl-x_L protein, which was

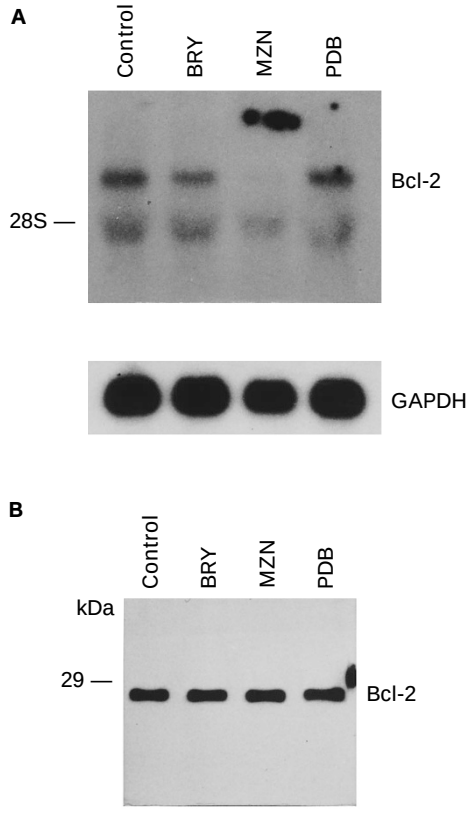

Figure 2 (**A**) Northern analysis of *bcl-2* mRNA expression. Following incubation of HL-60 cells with 10 nM bryostatin 1, PDB, or MZN, total cellular RNA was extracted, subjected to electrophoresis, and hybridized with a radiolabeled *bcl-2* probe as described in the text. The housekeeping gene GAPDH was analyzed in parallel. (**B**) Western analysis of Bcl-2. Total cellular protein was extracted, electrophoresed, and probed with an antibody to Bcl-2. After reaction with secondary antibody, blots were developed using chemiluminescence as described in the text. A representative study is shown; two additional experiments yielded equivalent results.

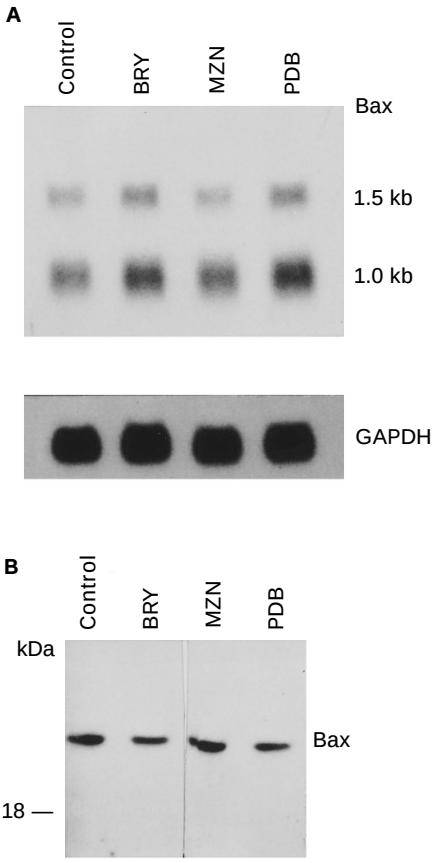

Figure 3 Northern (**A**) and Western (**B**) analysis of Bax expression in HL-60 cells exposed to 10 nM bryostatin 1, PDB, or MZN for 24 h. Two additional studies yielded equivalent results.

initially present in low abundance in these cells. For example, bryostatin 1-treated cells displayed Bcl-x_L levels equivalent to 119 ± 29% of controls ($p \geqslant 0.05$). Equivalent results were obtained when *bcl-x$_L$* message was monitored (data not shown). Levels of the pro-apoptotic short form (Bcl-x_S) were undetectable in both control and PKC activator-treated cells.

A different pattern emerged when expression of the anti-apoptotic gene Mcl-1 was monitored (Figure 5). Cells exposed to MZN for 24 h exhibited an increase in *mcl-1* mRNA, which appeared predominantly in the smaller 2.5 kb species, consistent with previous reports associating increased expression of this gene with a more differentiated state (Kozopas *et al*, 1993). More modest increases in message were also noted in cells exposed to bryostatin 1 and PDB, despite the relative failure of these agents to trigger a differentiation program in this subline. Similarly, MZN, and, to a lesser extent, BRY and PDB induced clear increases in levels of Mcl-1 protein. Thus, the ability of bryostatin 1 to potentiate ara-C-induced apoptosis could not be attributed to reduced expression of the anti-apoptotic gene Mcl-1.

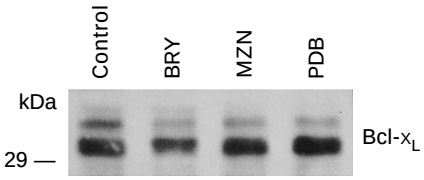

Figure 4 Western analysis of Bcl-x_L protein in HL-60 cells exposed to bryostatin 1, PDB, or MZN was performed as described in the text. Expression of Bcl-x_S protein was below the level of detection of this assay. Two additional studies yielded equivalent results.

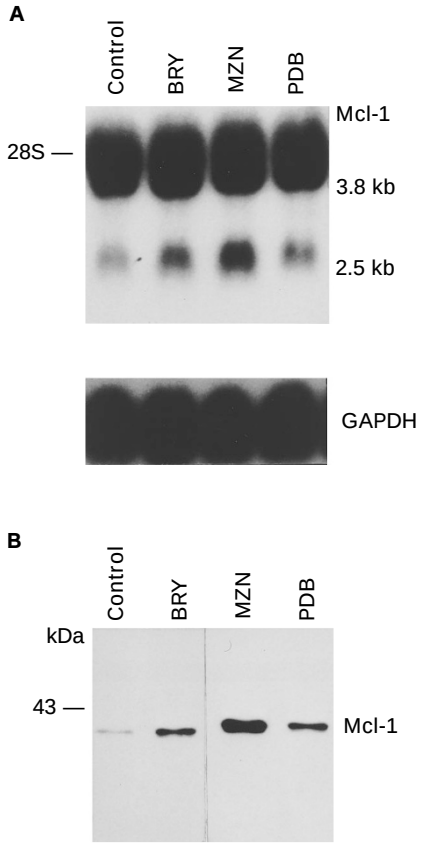

Figure 5 Northern (**A**) and Western (**B**) analysis of Mcl-1 expression in HL-60 cells exposed to 10 nM bryostatin 1 (BRY), PDB, or MZN for 24 h. A representative study is shown; two additional experiments yielded equivalent results.

Previous studies have demonstrated that bryostatin 1 blocks certain phorboid-mediated actions that it does not itself exert, including induction of leukemic cell differentiation (Kraft *et al*, 1986). Co-administration of 10 nM bryostatin 1 prevented 10 nM MZN from triggering HL-60 cell maturation, as reflected by antagonism of plastic adherence, while the combination continued to potentiate ara-C-mediated apoptosis (Figure 6A; Table 1). Thus, the actions of bryostatin 1 were dominant to those of MZN with respect to both maturation and apoptosis. However, when bryostatin 1 was combined with MZN, up-regulation of Mcl-1 protein was at least as great as that observed with MZN alone (Figure 7), indicating a dissociation between leukemic cell differentiation and Mcl-1 expression. This finding also indicates that increased expression of Mcl-1 protein is insufficient to prevent bryostatin 1 from potentiating ara-C-mediated apoptosis. As might be predicted from results illustrated in Figures 2–4, levels of Bcl-2, Bax, and Bcl-x_L protein remained essentially unchanged following combined exposure to bryostatin 1 and MZN. Lastly, it has recently

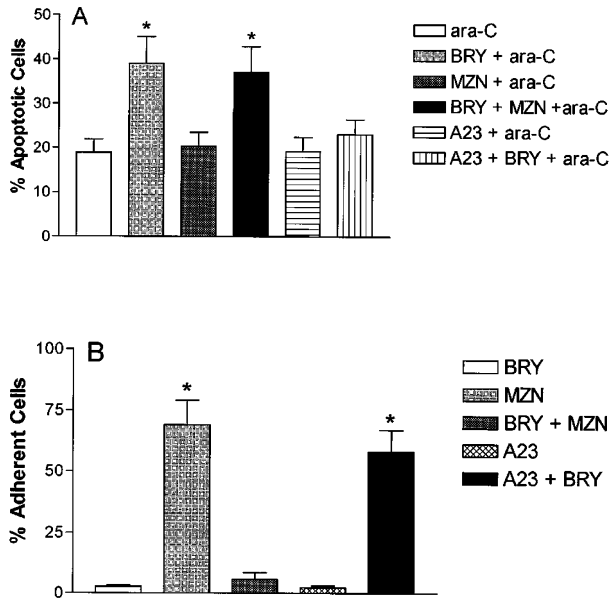

Figure 6 Cells were exposed to 10 nM BRY ± either 10 nM MZN or 250 nM A23187 (A23) for 24 h followed by a 6-h incubation with 10 μM ara-C (**A**). The percentage of apoptotic cells were then determined as described in the text. Alternatively, cells were exposed to bryostatin 1 ± MZN or A23187 for 24 h after which the percentage of adherent cells determined as described in Materials and Methods. Values represent the means for three separate experiments performed in triplicate ± S.D. *=significantly greater than values for ara-C alone (**A**) or BRY (**B**); $P \leqslant 0.02$.

been reported that administration of the calcium ionophore A23187 partially restores the ability of bryostatin 1 to induce differentiation in HL-60 cells, but, in so doing, antagonizes its capacity to potentiate ara-C-induced apoptosis (Grant *et al*, 1995). Thus, when 10 nM bryostatin 1 was combined with 250 nM A23187, plastic adherence was dramatically increased, while augmentation of ara-C-related apoptosis was reduced (Figure 6B; Table 1). Under these conditions, Mcl-1 protein expression was substantially increased, whereas expression of Bcl-2, Bax, and Bcl-x_L proteins remained essentially unchanged, a pattern identical to that observed in cells exposed to MZN alone (Figure 7).

Finally, in view of recent evidence that taxol-induced apoptosis in human leukemic lymphoblasts is associated with phosphorylation (and presumably inactivation) of the Bcl-2 protein (Haldar *et al*, 1995b), an attempt was made to determine if bryostatin 1 might act through a similar mechanism (Figure 8). In these studies, incubations were carried out in the presence of the phosphatase inhibitor sodium orthovanadate which inhibits dephosphorylation of the Bcl-2 protein. Exposure of cells to ara-C alone did not result in a modified Bcl-2 species. However, treatment of cells with 250 nM taxol for 6 h produced a mobility shift in the Bcl-2 protein, manifested by the appearance of a distinct, slowly migrating band. Several earlier studies have demonstrated that such a band corresponds to a phosphorylated Bcl-2 protein (Haldar *et al*, 1995b; Blagosklonny *et al*, 1996b). Although cells exposed to 10 nM bryostatin 1, whether or not they were subsequently treated with 10 μM ara-C for 6 h, failed to exhibit a separate hyperphosphorylated Bcl-2 species, they did display a diffuse widening of the protein band. Furthermore, coadministration of 10 μg/ml cyclohexamide, a concentration previously reported to inhibit protein synthesis in HL-60 cells by 90% (Kaufmann, 1989), did not prevent bryostatin 1-induced modification of the Bcl-2 protein (data not shown). These findings suggest that changes in the Bcl-2 protein accompanying bryostatin 1 and ara-C-associated apoptosis are qualitatively distinct from those that occur in cells exposed to taxol, and are not dependent upon protein synthesis.

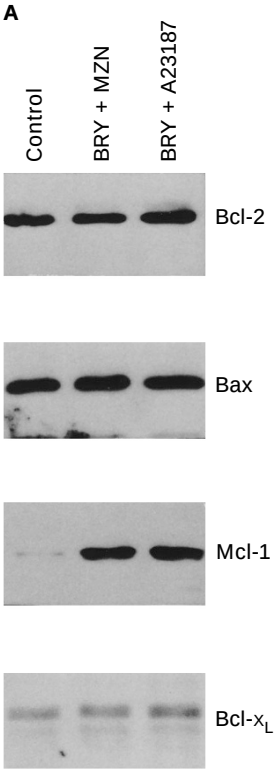

Figure 7 Western analysis of Bcl-2, Bax, Bcl-x_L, and Mcl-1 expression in cells exposed for 24 h to bryostatin 1 (10 nM) ± either 10 nM MZN or 250 nM A23187. A representative study is shown; two additional experiments yielded equivalent results.

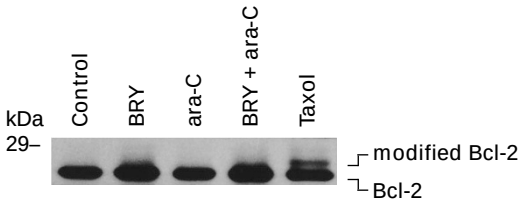

Figure 8 Western analysis of Bcl-2 protein obtained from control cells (lane 1); cells exposed to 10 nM bryostatin 1 for 24 h (lane 2); 10 μM ara-C for 6 h (lane 3); 10 nM bryostatin 1 for 24 h followed by ara-C (lane 4); 250 nM taxol for 6 h (lane 5). A representative study is shown; two additional experiments yielded equivalent results.

Discussion

The results of this study indicate that the ability of the PKC activator bryostatin 1 to potentiate ara-C-mediated apoptosis in the human myeloid leukemia cell line HL-60 involves factors other than or in addition to alterations in levels of expression of Bcl-2 and homologous proteins. Previous studies have demonstrated that a complex relationship exists between hematopoietic cell differentiation and expression of *bcl-2* and related genes. For example, in the murine leukemia cell line M1, induction of differentiation by dexamethasone and IL-6 over 2–3 days down-regulated *bcl-2* and up-regulated *bax* mRNA levels (Lotem and Sachs, 1995), although both agents increased *bcl-x_L* expression. In human hematopoietic cells, maturation is associated with a decline in both Bcl-2 (Delia *et al*, 1992) and Bcl-x_L levels (Benito *et al*, 1996). Moreover, human leukemic cells (HL-60) induced to undergo terminal differentiation (e.g. by all-*trans* retinoic acid) die via apoptosis (Martin *et al*, 1990), an event accompanied by Bcl-2 down-regulation (Hu *et al*, 1996). Moreover, when HL-60 cells are genetically modified to overexpress Bcl-2, they retain their capacity to undergo maturation in response to retinoic acid and tumor promoters such as PMA, although the onset of apoptosis may be delayed (Naumovski and Cleary, 1994; Park *et al*, 1994). Collectively, these findings raise the possibility that terminally differentiated cells undergo apoptosis as a consequence of reduced Bcl-2 expression. Analogously, down-regulation of *bcl-2* mRNA in HL-60 and U937 monocytic leukemia cells by ionizing radiation, tumor necrosis factor-α, and ceramide has recently been postulated

to contribute to induction of apoptosis by these agents (Chen *et al*, 1995). In this context, it is noteworthy that lipid second messengers have been implicated in leukemic cell differentiation and resulting apoptosis (Ohta *et al*, 1995). Since PKC activators, including bryostatin 1, are potentially capable of inducing leukemic cell maturation (Stone *et al*, 1988), it is tempting to speculate that the modulatory effects of these agents on drug-related apoptosis reflect alterations in the expression and/or function of Bcl-2 and related proteins.

The results of the present study indicate that the actions of bryostatin 1 and other PKC activators cannot simply be explained by perturbations in the expression of members of the *bcl-2* family, at least as far as ara-C-induced apoptosis is concerned. In fact, several earlier studies have shown that induction of leukemic cell differentiation (e.g., by PMA or DMSO) can reduce, rather than increase, drug-induced apoptosis (Solary *et al*, 1993; Del Bino *et al*, 1994). Difficulties in reconciling the latter observation with differentiation-related reductions in *bcl-2* expression may reflect the disparate time frames of such studies. For example, the majority of terminally differentiating cells undergoing apoptosis do so several days after exposure to differentiating agents (Martin *et al*, 1990). In contrast, in the reports alluded to above, as well as the present studies, cells were exposed to cytotoxic drugs within 24 h of addition of the putative differentiation agent. Another consideration relevant to such short exposure intervals is the half-life of the Bcl-2 protein, which has been estimated to be 24–48 h in leukemic cells (Hu *et al*, 1996; Blagsklonny *et al*, 1996a). In this context, MZN, which is a potent inducer of maturation in the HL-60 subline used in these studies (Jarvis *et al*, 1994b), led to down-regulation of *bcl-2* mRNA levels after 24 h of exposure. This finding is consistent with results obtained in U937 monocytic leukemic cells exposed to TNF, ionizing radiation, and ceramide (Chen *et al*, 1995). It is also compatible with the results of another recent report in which decreased expression of *bcl-2* mRNA in human erythroleukemia cells (TF-1) subjected to growth factor deprivation was found to be PKC-dependent (Rinaudo *et al*, 1995). However, in the present study, down-regulation of *bcl-2* mRNA was not accompanied by a significant reduction in Bcl-2 protein, which could help to explain why MZN failed to antagonize ara-C-induced apoptosis. Given the relatively long-half life of Bcl-2 protein, and the fact that bryostatin 1 is not an effective inducer of differentiation in this HL-60 subline (Jarvis *et al*, 1994b), it is not surprising that facilitation of apoptosis by a short exposure to bryostatin 1 does not involve reduced expression of Bcl-2. An alternative possibility is that bryostatin 1 might act by altering ratios of pro- and anti-apoptotic proteins, which are known to be differentially regulated in hematopoietic cells over a 48 to 72 h interval (Gottschalk *et al*, 1996). For example, activation of peripheral blood lymphocytes by various stimuli has been associated with the rapid modulation of Bcl-x_L but not Bcl-2 expression (Boise *et al*, 1995). However, neither bryostatin 1 nor its tumor-promoting counterparts altered expression of Bax or Bcl-x_L proteins at the 24 h interval. Collectively, these findings indicate that a reduction in Bcl-2 protein, or in Bcl-2/Bax or Bcl-x_L/Bax ratios, cannot be invoked to account for potentiation of ara-C-induced apoptosis by bryostatin 1.

The results of this study differ from those of a recent report in which a 24-h exposure of a large cell lymphoma cell line to bryostatin 1 resulted in down-regulation of Bcl-2 expression, as determined by immunohistochemical analysis (Maki *et al*, 1995). This capacity may reflect the higher bryostatin 1 concentration used in the latter study (e.g., 200 versus 10 nM); alternatively, it might stem from inherent differences in the responses of myeloid versus lymphoid cells to this agent. Since bryostatin 1 is capable of triggering a maturation program in cells of lymphoid origin (Drexler *et al*, 1989), in contrast to the HL-60 line used in these studies (Jarvis *et al*, 1994b), it is possible that down-regulation of Bcl-2 is closely linked to specific differentiation-related events. The observation that MZN, which is an effective inducer of HL-60 cell maturation, did not down-regulate Bcl-2 protein expression, at least within a 24-h time frame, highlights potential pitfalls in attempting to extrapolate findings in lymphoid neoplasms to those of myeloid origin. Moreover, evidence that 1,25-dihydroxyvitamin D_3 opposes ara-C-mediated apoptosis in HL-60 cells despite down-regulating Bcl-2 expression (Xu *et al*, 1993), indicates that differentiation-related reductions in Bcl-2 levels do not necessarily lead to increased drug susceptibility.

Of the proteins examined in this study, perturbations in expression of the Mcl-1 by PKC activators were the most pronounced. Mcl-1 expression was originally shown to increase early in ML-1 leukemia cells induced to differentiate by TPA (Kozopas *et al*, 1993). Studies in yeast suggest that Mcl-1 may function analagously to Bcl-2 in suppressing the death-promoting actions of Bax (Bodrug *et al*, 1995). Evidence of an anti-apoptotic action is based on the observations that expression of Mcl-1 correlates with the survival of peripheral blood lymphocytes (Lomo *et al*, 1996), and that overexpression of Mcl-1 in Chinese hamster ovary cells protects them from *c-myc*-driven apoptosis (Reynolds *et al*, 1994). On the other hand, overexpression of Mcl-1 in IL-3-dependent myeloid cells (32D.3 and FL5.12) failed to protect them from growth factor deprivation-induced apoptosis, despite its capacity to neutralize Bax-mediated cytotoxicity in yeast (Bodrug *et al*, 1995). It seems likely, therefore, that the role of Mcl-1 in regulating apoptosis is both cell line- and stimulus-dependent, and that its capacity to neutralize Bax is weaker than that of Bcl-2. Although the failure of MZN to potentiate ara-C-mediated apoptosis was temporarily associated with increased Mcl-1 expression, bryostatin 1, which was capable of augmenting ara-C-induced cell death, also up-regulated Mcl-1, albeit to a limited extent. Moreover, the combination of bryostatin 1 and MZN, which fully potentiated ara-C-mediated apoptosis, resulted in a further increase in Mcl-1 expression. Based upon these findings, it appears unlikely that Mcl-1 acts in this system to oppose ara-C-induced apoptosis, or that changes in its expression account for the actions of bryostatin 1. It is nevertheless interesting that while bryostatin 1 opposed MZN-induced differentiation in these cells, it did not prevent the accompanying increase in Mcl-1 expression; in fact, it had

the opposite effect. This would suggest that up-regulation of Mcl-1 is linked to very early, pre-commitment maturation events which are dissociable from those responsible for terminal differentiation.

Despite the lack of a clear relationship between bryostatin 1-mediated potentiation of ara-C-related apoptosis and reduction in Bcl-2 expression, it remains possible that alterations in Bcl-2 function stemming from perturbations in PKC activity may contribute to observed effects. For example, PKC has been implicated in interactions between *bcl-2* and both ras and Raf-1 (Chang and Faller, 1995). Moreover, bryostatin 1-mediated phosphorylation of Bcl-2 on serine residues in IL-3-dependent hematopoietic cells has been invoked to account for the ability of this agent to prevent growth factor deprivation-induced apoptosis (May *et al*, 1994). In contrast, taxol-induced apoptosis in neoplastic cells has also been temporally associated with Bcl-2 phosphorylation, possibly through a Raf-1-dependent pathway (Blagosklonny *et al*, 1996b), raising the possibility that under certain circumstances phosphorylation of Bcl-2 may interfere with its protective effects (Haldar *et al*, 1995a and b). In this regard, the observation that treatment of HL-60 cells with taxol produced a distinct mobility shift in the Bcl-2 protein, whereas the combination of bryostatin 1+ara-C resulted in a broader, more diffuse change in Bcl-2 migration, suggests that induction of apoptosis by these agents involves separate mechanisms, at least as far as Bcl-2 is concerned. While it is possible that bryostatin 1 might regulate Bcl-2 expression at the translational level, this appears unlikely in view of (1) the inability of cyclohexamide to modify bryostatin 1's effect; and (2) the relatively long half-life of the Bcl-2 protein, which has been reported to be as long as 24–48 h in leukemic cells (Hu *et al*, 1996; Blagosklonny *et al*, 1996a). Discrepancies between this and a previous report (May *et al*, 1994), in which bryostatin 1-mediated phosphorylation of Bcl-2 was associated with inhibition of apoptosis, may stem from differences in the response of normal and leukemic cells to this agent. Alternatively, they could reflect qualitative, rather than quantitative, changes in Bcl-2 phosphorylation status following bryostatin 1 exposure. Finally, the possibilities that Bcl-2 phosphorylation plays divergent roles in growth factor deprivation-mediated (May *et al*, 1994) versus drug-induced apoptosis (Haldar *et al*, 1995a and b), or that bryostatin 1 modulates interactions between Bcl-2 and other homologs (e.g. Bag, Bad, A1, Bax, etc.), remain to be explored. Metabolic studies of Bcl-2 phosphorylation by bryostatin 1 are currently in progress, and are designed to address some of these questions.

In summary, the present findings indicate that the capacity of bryostatin 1 to potentiate ara-C-induced apoptosis in human myeloid leukemia cells involves factors other than quantitative changes in the expression of Bcl-2, Bax, Bcl-x_L, or Mcl-1, and raise the possibility that qualitative modifications in the Bcl-2 protein, such as phosphorylation status, may play a role in this phenomenon. They also demonstrate that reductions in *bcl-2* mRNA levels following induction of leukemic cell differentiation need not be associated with a parallel decrease in protein expression, at least over a limited time frame, and that up-regulation of Mcl-1 may occur in the absence of cellular maturation. Lastly, attention has recently focused on the opposing influence of the PKC and sphingomylinase pathways in the regulation of survival of leukemic (Jarvis *et al*, 1994a) and other cell types (Chmura *et al*, 1996). In addition, it is known that ceramide-induced apoptosis may be opposed, at least in part, by overexpression of Bcl-2 (Martin *et al*, 1995). Since bryostatin 1 is a potent down-regulator of PKC activity (Isakov *et al*, 1993), interactions between this agent and members of the Bcl-2 family may be indirect, mediated through an increase in the relative activity of the pro-apoptotic ceramide versus the protective PKC signal transduction pathway. These considerations may be particularly pertinent to an agent such as ara-C, which is known to lead to generation of both ceramide (Strum *et al*, 1994) and diacylglycerol (Kucera and Capizzi, 1992) in leukemic cells. Studies designed to address these questions are currently in progress.

Materials and Methods

Cells

The human promyelocytic leukemia cell line, HL-60, was derived from a cell line originally described (Gallagher *et al*, 1979). Cells were maintained in RPMI 1640 (phenol red-free formulation; Sigma) supplemented with 0.2% sodium bicarbonate, 1.0% sodium pyruvate, non-essential amino acids, L-glutamine, PSN antibiotic mix (MediaTech, Herndon, VA) and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT). Cultures were kept under a fully humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cultures were routinely screened for mycoplasma contamination with a rapid hybridization assay for mycoplasma RNA (Gen-Probe, San Diego, CA) and consistently found to be mycoplasma-free. Cell densities were determined using a model ZB1 Coulter Counter (Hialeah, FL) and cell viability was assessed by trypan blue exclusion. Cells in log-phase growth (cell density=2.5×10^5 cells/ml) were incubated with the designated concentrations of drug and maintained as described above. Experimental incubations were terminated by pelleting cells at $400 \times g$ for 10 min followed by aspiration of the media. Cell pellets were subsequently prepared for procedures described below.

Drugs

PDB and MZN were purchased from LC Laboratories (Woburn, MA), formulated in dimethylsulfoxide (DMSO; Sigma Chemicals, St. Louis, MO), and stored under light-protected conditions at −20°C prior to use. Taxol and A23187 were purchased from Sigma Chemicals, stored as a dry powder at −20°C in a dark container pending use, and formulated in DMSO and water respectively. Cyclohexamide was purchased from Sigma, stored in sealed containers at −20°C, and formulated immediately prior to use in ethanol. The final concentration of ethanol was in all cases $\leqslant$0.01%. Sodium orthovanadate was purchased from Aldrich Biochemicals, Milwaukee, WI, and formulated in sterile water. Bryostatin 1 was provided as a lyophilized powder by Dr. James Pluda, Cancer Treatment and Evaluation Branch, NIH/NCI, stored at −20°C, and also dissolved in DMSO. After appropriate dilutions, the final concentration of DMSO in all test conditions was $\leqslant$0.05%, a level found to have no discernible effect on apoptosis or Bcl-2 expression. A23187 (calcium ionophore) was purchased from Sigma, stored as a dry powder at −20°C, and formulated in sterile water prior to use.

Morphological assessment of apoptosis

Following treatment of cells, cytocentrifuge preparations were made utilizing a CYTO-TEK centrifuge. Slides were stained using a modified Wright-Giemsa stain (Diff-Quik, Baxter Healthcare, Miami FL) according to the manufacturer's instructions, and viewed at 1000× magnification with the aid of an Olympus microscope. The percentage of apoptotic cells, defined as those exhibiting the characteristic features of cell shrinkage, nuclear condensation, and the formation of membrane-bound apoptotic bodies, was determined by evaluating at least 500 cells/condition in triplicate, as previously described (Jarvis *et al*, 1994).

DNA fragmentation

A previously described quantitative spectrofluorometric assay was employed to monitor low molecular weight DNA fragmentation in cells after drug exposure (Jarvis *et al*, 1995c). Briefly, following 24 h incubation with the designated concentration of bryostatin 1, with or without 1.5 μM AS101, cells (3×10^6/condition) were exposed to 10 μM ara-C for an additional 6 h. The cells were then pelleted by centrifugation at 400 × *g* for 7 min, washed with fresh medium, and the pellets lysed overnight in 5 mM Tris (pH 7.4) buffer containing 0.1% Triton X-100, and 20 mM EDTA. They were then subjected to high-speed centrifugation at 44 000 × *g* for 50 min at 4°C after which the supernatant was extracted and diluted in 3 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 containing 1.0 μg/ml *bis*benzimide trihydrochloride (Hoechst-33258; Sigma) in order to monitor the presence of non-sedimenting low molecular weight DNA fragments. Determination of net fluorescence in each sample (λex=365, λem=460) was used to calculate DNA values following comparison to highly purified standard preparations of calf thymus DNA. We have previously shown that the degree of DNA fragmentation documented by this assay correlates closely with qualitative results obtained by agarose gel electrophoresis, as well as with the percentage of cells displaying the characterisic morphologic features of apoptosis (Jarvis *et al*, 1994b).

Cell adherence

Logarithmically growing cells were placed in 25 cm^2 plastic tissue culture flasks (Corning, Corning NY), to which were added various agents at the designated concentrations. Subsequently, the density of cells in suspension was determined utilizing a hematocytometer. The sides of the flasks were then scraped with a rubber policeman to permit detachment of adherent cells, and the cells dispersed prior to repeat density determinations. The percentage of adherent cells was expressed as a percentage relative to the total cell population.

Northern analysis

Total cellular RNA was isolated from 1×10^7 cells using RNA STAT-60 reagent and protocol (Tel-Test 'B', Inc., Friendswood, TX). Total RNA (15 mg) was then separated on a 1.0% agarose/formaldehyde gel as previously described (Jarvis *et al*, 1994b). The RNA was blotted onto nylon (Schleicher and Schuell, Keene, MD) by downward capillary transfer for 16 h and then cross-linked to the nylon by baking at 80°C for 2 h. The blots were then hybridized as previously described (Jarvis *et al*, 1994c) with a Bcl-2 cDNA probe (provided by Michael Cleary, Stanford University, Stanford, CA), and Bax, Bcl-x and Mcl-1 cDNA probes (Krajewski *et al*, 1994a and 1994b). To ensure equal loading and transfer, the blots were subsequently stripped and hybridized with cDNA probe for the housekeeping gene, GAPDH. Probes were nick-translated with [α-^{32}P]dCTP, (3000 Ci/mM) (New England Nuclear, Boston, MA) using the Nick Translation System (Life Technologies, Grand Island, NY). The blots were washed extensively in 0.2 × SSC/0.1% SDS (1 × SSC=150 mM NaCl, 15 mM sodium citrate) at 65°C, placed on Fuji RX film (Fuji Photo Film Co, Japan) with intensifying screens, and exposed at −90°C. Autoradiographs were quantitated via laser densitometry (Molecular Dynamics, Sunnyvale, CA), and values determined following normalization for expression of GAPDH

Western analysis

Whole cell pellets (1×10^7 cells) were washed twice in PBS. Cell pellets were then resuspended in 100 μl PBS and lysed by the addition of 100 μl 2× loading buffer (1 ×=30 mM Tris-base, pH 6.8, 2% SDS, 2.88 mM β-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue). Lysates were sonicated, boiled for 10 min, centrifuged at 15 600 × *g* for 2 min, and quantified using Coomassie protein assay reagent (Pierce, Rockford, IL). Equal quantities of protein (10 μg) were separated by SDS–PAGE (5% stacker and 12% resolving) and electroblotted to nitrocellulose. The blots were stained in 0.1% amido black and destained in 5% acetic acid to ensure transfer and equal loading. The blots were then blocked in PBS-Tween (0.05%) and 5% non-fat dry milk for 1 h at 22°C. They were subsequently incubated in fresh blocking solution with Bcl-2 primary antibody (Ab 1; 1:1000, DAKO Corporation, Carpinteria, CA) Bax (1:1000; Santa Cruz Biotechnology Inc, Santa Cruz, CA), Mcl-1 (1:1000; Krajewski *et al*, 1994a), or Bcl-x (1:1000; Santa Cruz) for 1 h at 22°C. Blots were washed 2 × 10 min in PBS-T and then incubated with a HRP-conjugated secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburgh, MD) for 1 h at 22°C. Blots were again washed 2 × 10 min in PBS-T and then developed by enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL) as per the manufacturer's instructions.

In studies involving taxol, samples were prepared exactly as described except that loading and transfer buffer contained the phosphatase inhibitor sodium orthovanadate (200 mM) to inhibit dephosphorylation of the Bcl-2 protein. Western analysis was otherwise performed as outlined above.

Quantitation of protein expression

Autoradiographs of Western blots were scanned using a Molecular Dynamics Personal Densitometer SI and quantified using Molecular Dynamics Image Quant software version 4.1. Changes in protein levels were determined by comparing the ratio of protein levels in treated and untreated cells and averaging results for 3–4 separate experiments.

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