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Agents that Down-Regulate or Inhibit Protein Kinase C Circumvent Resistance to 1-β-D-Arabinofuranosylcytosine-Induced Apoptosis in Human Leukemia Cells that Overexpress Bcl-2

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SUMMARY

The effects of the non-tumor-promoting protein kinase C (PKC) activator bryostatin 1 and the PKC inhibitors staurosporine and UCN-01 were examined with respect to modulation of 1-[β-D-arabinofuranosyl]cytosine (ara-C)-induced apoptosis in human myeloid leukemia cells (HL-60) overexpressing the antiapoptotic protein Bcl-2. HL-60/Bcl-2 cells displayed a 5-fold increase in Bcl-2 protein compared with empty-vector counterparts (HL-60/pCEP4) but comparable levels of Bax, Mcl-1, and Bcl-x_L. After exposure to an equimolar concentration of ara-C (10 μM for 6 hr), HL-60/Bcl-2 cells were significantly less susceptible to apoptosis, DNA fragmentation, and loss of clonogenicity than HL-60/pCEP4 cells. The protective effect of increased Bcl-2 expression was manifested by a failure of ara-C to induce activation/cleavage of the Yama protease (CPP32; caspase-3) and degradation of one of its substrates, poly(ADP-ribose)polymerase to an 85-kDa cleavage product. When HL-60/Bcl-2 cells were preincubated with bryostatin 1 (10 nM; 24 hr) or coincubated with either staurosporine (50 nM; 6 hr) or UCN-01 (300 nM; 6 hr) after a 1-hr preincubation, exposures that exerted minimal effects alone, ara-C-induced apoptosis and DNA fragmentation were restored to levels equivalent to, or greater than, those observed in empty-vector controls. These events were accompanied by restoration of the ability of ara-C to induce CPP32 cleavage and activation, poly(ADP-ribose) polymerase degradation, and inhibition of colony formation. Western analysis of Bcl-2 protein obtained from overexpressing cells treated with bryostatin 1, staurosporine, or UCN-01 revealed the appearance of a slowly migrating species and a general broadening of the protein band, effects that were insensitive to the protein synthesis inhibitor cycloheximide. Alterations in Bcl-2 protein mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis were reversed by treatment of lysates with alkaline phosphatase or protein phosphatase 2A; actions of the latter were blocked by the specific phosphatase inhibitor okadaic acid. *In vivo* labeling studies of Bcl-2 protein demonstrated increased incorporation of [$^{32}PO_4$]orthophosphate in drug-treated cells. Last, phosphorylated Bcl-2 failed to display decreased binding to the proapoptotic protein Bax. Collectively, these findings indicate that bryostatin 1, which down-regulates PKC, and staurosporine and UCN-01, which directly inhibit the enzyme, circumvent resistance of Bcl-2-overexpressing leukemic cells to ara-C-induced apoptosis and activation of the protease cascade. They also raise the possibility that modulation of Bcl-2 phosphorylation status contributes to this effect.

The proto-oncogene *bcl-2* encodes a cytoprotective 26-kDa protein (Bcl-2) intimately involved in the regulation of programmed cell death, or apoptosis. Bcl-2 is a member of a family of genes homologous to the *Caenorhabditis elegans* death (*ced*) family (*ced-3*, *ced-4*, and *ced-9*) and is believed to represent the mammalian homolog of *ced-9* (reviewed in Ref. 1). The product of the *bax* gene (Bax), another family member, forms homodimers that have been implicated in the apoptotic response. The ability of Bax to induce cell death is antagonized by dissociation of Bax/Bax homodimers to form Bcl-2/Bax heterodimers (2). Bcl-x, another Bcl-2 homolog, gives rise through alternative splicing to a long form (Bcl-x_L) and a short form (Bcl-x_s). Bcl-x_L, like Bcl-2, opposes apoptosis, whereas Bcl-x_s acts like Bax to promote cell death (3). During the past several years, other Bcl-2-related proteins

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ABBREVIATIONS: ara-C, 1-β-D-arabinofuranosylcytosine; PMA, phorbol-12-myristate-13-acetate; PKC, protein kinase C; DMSO, dimethylsulfoxide; PP2A, protein phosphatase 2A; OA, okadaic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PARP, poly(ADP-ribose)polymerase; PBS-T, phosphate-buffered saline/Tween 20; ICE, interleukin-1β counting enzyme.

have been identified, including Mcl-1, A1, Bad, and Bag (4). It is possible that the susceptibility of cells to apoptosis depends on complex interactions between proapoptotic and antiapoptotic proteins.

The role that Bcl-2 plays in the regulation of apoptosis has particular significance for hematological malignancies, most notably, leukemia. For example, leukemic patients whose cells express high levels of Bcl-2 respond poorly to chemotherapy compared with patients whose blasts express low levels of this protein (5). In preclinical models, leukemic cell lines genetically engineered to overexpress Bcl-2 (or Bcl-x_L) display resistance to a broad range of cytotoxic agents, including ara-C (6, 7). In a recent large prospective series involving primary leukemic blast specimens, Banker *et al.* (8) reported that cells expressing high levels of Bcl-2 were resistant to apoptosis after *ex vivo* exposure to ara-C. Collectively, these findings suggest that Bcl-2 may represent an important determinant of chemosensitivity in leukemia.

The protective actions of Bcl-2 have been related to prevention of activation of ICE-like proteases, referred to as caspases, that involved in the degradation phase of apoptosis. Accumulating evidence suggests that the mammalian *ced-3* equivalent may not be ICE (caspase-1) itself but instead one or more members of a family of aspartate-directed cysteine proteases, including Nedd-2/ICH1 (caspase-2), Yama/CPP32/apopain (caspase-3), ICH2 (caspase-4), Mch2 (caspase-6), and ICE-LAP3/Mch3 (caspase-7) (9). The Yama protease CPP32 (caspase-3), whose substrates include PARP and the U1 small ribonucleoprotein, has been found to be activated in leukemic cells exposed to chemotherapeutic drugs such as ara-C (7). Recently, it has been shown that Jurkat cells overexpressing Bcl-2 and Bcl-x_L are resistant to staurosporine-induced CPP32 activation and PARP cleavage, thereby localizing the Bcl-2 and Bcl-x_L proteins in the cell death hierarchy (10). In an analogous manner, human myeloid leukemia cells overexpressing Bcl-2 display reduced CPP32 activation and PARP degradation after exposure to ara-C (6, 7). The mechanism linking Bcl-2-overexpression to resistance to caspase activation remains the subject of intense interest.

Previous studies from this laboratory have shown that chronic pretreatment of human myeloid leukemia cells (HL-60) with the PKC activator bryostatin 1 sensitizes them to ara-C-mediated apoptosis (11), a process temporally related to down-modulation of PKC activity (12). In view of evidence that (a) tumor-promoting phorboids such as PMA acutely antagonize apoptosis in hematopoietic cells (13), (b) acute exposure to PKC inhibitors promotes this process (14), and (c) PKC inhibitors potentiate drug-induced apoptosis (15), it is possible that PKC acts in some way to protect leukemic cells from lethal insults. In this context, the results of several recent reports suggest that the action of the Bcl-2 protein may be regulated, at least in part, through a PKC-dependent pathway (16, 17). The purpose of the current report was to determine whether, and to what extent, bryostatin 1 and two PKC inhibitors, staurosporine and its 7-hydroxy derivative (UCN-01), could increase the sensitivity of Bcl-2-overexpressing leukemic cells to ara-C-mediated apoptosis. Our results indicate that each of these agents restores the ability of ara-C to trigger an apoptotic response, including activation of the apoptotic protease cascade, in otherwise resistant cells. Furthermore, this capacity is associated with phosphorylation of the Bcl-2 protein.

Materials and Methods

Cells. The human promyelocytic leukemia cell line HL-60 was derived from a patient with acute promyelocytic leukemia as described previously (11). Cells were cultured in RPMI 1640 medium supplemented with sodium pyruvate, minimal essential medium, essential vitamins, L-glutamate, penicillin and streptomycin, and 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT). They were maintained in a 37°/5% CO_2, fully humidified incubator and passed twice weekly.

A Bcl-2-overexpressing HL-60 cell variant was generated using a commercially available vector (pCEP4; InVitrogen, Carlsbad, CA) and human Bcl-2 cDNA (provided by M. Cleary, Stanford University). Then, 25 μg of plasmid DNA was electroporated in 400 μl of cold 1× RPMI containing 1×10^7 HL-60 cells. Electroporation was performed in a 0.4-cm cuvette using a BTX electromanipulator 600 set at 264 V, 750 mF, and 480 W. Cells were then diluted to a concentration of 1×10^6 cells/ml in 1× RPMI plus 10% FBS and incubated for 48 hr in a 37°/5% CO_2, fully humidified incubator. Hygromycin B (Boehringer-Mannheim Biochemicals) was added to achieve a final concentration of 400 mg/ml, after which cells were diluted to 1×10^5/ml, and 200-μl aliquots were plated onto 96-well plates. Single-cell clones were obtained by limiting dilution and subsequently analyzed for an increase in Bcl-2 mRNA and protein expression relative to identically cloned empty vector controls (HL-60/pCEP4). The HL-60/Bcl-2 line was used for all experiments; for comparison, another Bcl-2-overexpressing clone (designated HL-60/Bcl-2-B5) was examined as indicated. A common HL-60 subline (F8) was used for all transfections.

Drugs and chemicals. The drug ara-C was purchased from Sigma Chemicals(St. Louis, MO) and maintained as a dry powder at −20°. It was diluted in PBS (1× = 137 mM NaCl, 2.7 mM KCl, 10.2 mM Na_2HPO_4, 1.76 mM KH_2PO_4) before use. Bryostatin 1 was provided by the Cancer Treatment and Evaluation Program (National Institutes of Health, Bethesda, MD) and stored desiccated at −20°. Additional material was furnished by Dr. G. R. Pettit (Cancer Research Institute, Arizona State University, Tempe, AZ). We have found the activity of these preparations to be identical. Bryostatin 1 was formulated in sterile DMSO (Sigma) and subsequently diluted in RPMI 1640 medium so the final concentration of DMSO was in all cases ≤0.05%. UCN-01 (7-hydroxystaurosporine) was provided by Dr. Edward Sauseville (Division of Cancer Treatment, National Cancer Institute). It was stored in light-protected vials at −20° and formulated in sterile water before use. Staurosporine, taxol, HA-1004, and cyclohexamide were purchased from Sigma; stored in light-protected containers at −−20°; and dissolved in DMSO before use. OA (Sigma) was stored at −20° and formulated in sterile water. PP-2A was purchased from Upstate Biotechnology (Lake Placid, NY) and dissolved in dilution buffer before use according to the provider's instructions.

Experimental format. A previously described experimental format was used (12, 15). Briefly, logarithmically growing HL-60/pCEP4 and HL-60/Bcl-2 cells (concentration, $\approx 3 \times 10^5$ cells/ml) were placed in 25-cm^2 plastic T-flasks containing 10 nM bryostatin 1 and incubated for 24 hr in a 37°/5% CO_2 incubator. At the end of the incubation period, 10 μM ara-C was added to the flasks, which were then placed back into the incubator for an additional 6 hr. After this period, cells were pelleted and subjected to analysis as described below. Alternatively, cells were exposed to the designated concentration of staurosporine or UCN-01 for 1 hr, after which 10 μM ara-C was added to the flasks for an additional 6 hr. We demonstrated previously that in the case of bryostatin 1 and staurosporine, such exposures are equally effective in potentiating ara-C-induced apoptosis in parental HL-60 cells (12, 15).

Cell morphology and apoptosis. Cytocentrifuge preparations were stained with Wright-Giemsa and viewed by light microscopy to evaluate features of cellular differentiation as well as apoptosis (i.e., cell shrinkage, nuclear condensation, formation of apoptotic bodies, and so on), as described previously (12). For the latter studies, the percentage of apoptotic cells was determined by evaluating ≥500 cells/condition in triplicate. We reported previously that the incidence of apoptosis determined by these morphological criteria correlates very closely with the degree of low-molecular-weight DNA fragmentation assayed quantitatively by spectrofluorometry (see below), and qualitatively with the amount of internucleosomal DNA fragmentation determined by agarose gel electrophoresis (12).

DNA fragmentation. Quantification of DNA fragmentation was monitored in lysates treated with bisbenzamide as outlined previously in detail (12).

Western analysis. Expression of Bcl-2, Bax, Bcl-x_L, Mcl-1, CPP32, and PARP protein was determined by Western analysis using minor modifications of a previously described method (18). After treatment, whole-cell pellets (1×10^7 cells/condition) were washed twice in PBS, resuspended in 50 μl of PBS, lysed by the addition of 50 μl of 2× Laemmli's solution (1× = 30 mM Tris base, pH 6.8, 2% SDS, 2.88 mM β-mercaptoethanol, 10% glycerol), and briefly sonicated. Homogenates were quantified using Coomassie protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein (20 μg) were boiled for 10 min, separated by SDS-PAGE (5% stacker and 10% 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. After blocking in PBS/Tween 20 (0.05%) and 5% nonfat dry milk for 1 hr at 22°, the blots were incubated in fresh blocking solution with an appropriate dilution of primary antibody (Bcl-2 1:1000; DAKO, Carpinteria, CA/Bcl-x 1:1000; Transduction Laboratories, Lexington, KY/Bax 1:1000; Santa Cruz, Santa Cruz, CA/Mcl-1 1:1000; provided by Dr. J. Reed, the Burnham Institute, La Jolla, CA) for 4 hr at 22°. For studies of CPP32, primary antibody (Transduction Laboratories, Lexington, KY) was used at a concentration of 1:2000; for PARP (BIOMOL Research Laboratories, Plymouth Meeting, PA), an antibody concentration of 1:10,000 was used. Antibodies to tubulin (Calbiochem, La Jolla, CA) were used at a concentration of 1:500. Blots were washed three times for 5 min in PBS-T and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Kirkegaard and Perry, Gaithersburg, MD) for 1 hr at 22°. Blots were again washed three times for 5 min in PBS-T and then developed by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Protease activity. The activity of the Yama protease (caspase-3) was determined using a commercially available kit (ApoAlert; Clontech, Palo Alto, CA) according to the manufacturer's specifications. This method uses a colorimetric assay to monitor cleavage of an Ac-DEVD-*p*-nitroanilide substrate, characteristic of the caspase-3 cleavage site (6). Values were expressed as the concentration of Ac-DEVD-p-nitroanilide cleaved over the course of a 1-hr incubation interval.

Cell cycle analysis. The cell cycle distribution of HL-60/pCEP4 and HL-60/Bcl-2 cells was compared using a previously described flow cytometric method (12).

PKC activity. Total cellular PKC activity was determined using a commercially available kit (GIBCO, Grand Island, NY) as described previously in detail (12).

Cell adherence. After exposure to the designated agents for 72 hr, the density of cells in suspension was determined using a hematocytometer. The sides of the flasks were then scraped with a rubber policeman to permit detachment of adherent cells, and the cells were dispersed before repeat density determinations. The percentage of adherent cells was then expressed relative to the total cell population.

Clonogenic assay. Colony formation by treated cells was determined using a previously described soft agar cloning assay (11).

***In vivo* labeling and immunoprecipitations.** For ^{35}S-methionine labeling, HL60-pCEP4 and HL-60/Bcl-2 cells were washed twice in labeling medium (RPMI 1640 with L-glutamine, without L-methionine, 10% dialyzed fetal calf serum; GIBCO BRL, Gaithersburg, MD) and incubated in this medium for 15 min. Cells were then adjusted to a concentration of 3×10^5 cells/ml in medium containing 0.125 mCi/ml Tran^{35}S Label (1299 Ci/mmol; ICN, Costa Mesa, CA). Cells were treated with 10 nM bryostatin 1 for 24 hr or with 300 nM UCN-01 or 50 nM staurosporine for 6 hr. The cells were washed with cold PBS and lysed in radioimmunoprecipitation assay buffer (1% Nonidet P-40, 0.5% Na deoxycholate, 0.1% SDS, 0.5 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, 0.5 mM Na orthovanadate in PBS) and passed through a 21-guage needle. Bcl-2 was immunoprecipitated from equivalent amounts of protein with 1 μg of a monoclonal anti-human Bcl-2 antibody (DAKO, Carpinteria, CA) and goat anti-mouse Dynabeads (Dynal, Oslo, Norway). Immune complexes were eluted from the magnetic beads by boiling in SDS sample buffer for 10 min and electrophoresed on a 12% SDS-PAGE. The gel was fixed, enhanced with Fluoro-Hance (Research Products International, Mount Prospect, IL), and analyzed by fluorography using Fuji RX X-ray film.

For ^{32}P-labeling, HL60/pCEP4 and HL60/Bcl-2 cells were washed twice in phosphate-free RPMI medium containing 10% dialyzed fetal calf serum and resuspended in 10 ml of this medium to a final density of 5×10^5 cells/ml. Inorganic [^{32}P]orthophosphate was added to each condition (1 mCi/ml), and samples were incubated at 37°. After 1 hr, cells were treated with 10 nM bryostatin, 300 nM UCN-01, or 50 nM staurosporine, and the incubation was continued at 37°. After a further 3 hr, cells were pelleted, washed once with phosphate-free RPMI, repelleted, and snap-frozen in liquid N_2. Cell lysis and homogenization were carried out in 1.5 ml of ice-cold solution [25 mM sodium β-glycerophosphate, pH 7.4 at 4°, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, 1 mM PMSF, 40 mg/ml pepstatin A, 25 mM sodium fluoride, 0.5 mM sodium orthovanadate, 0.5 mM sodium pyrophosphate, 0.05% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 0.1% (v/v) 2-mercaptoethanol], with gentle trituration using a P1000 pipet to lyse the cells, and the homogenate was clarified by centrifugation. Bcl-2 was immunoprecipitated (12 hr, 4°) from equivalent amounts of clarified homogenate protein with 1 μg of a monoclonal anti-human Bcl-2 antibody coupled to Dynabeads as described above. Immunoprecipitates were sequentially washed (1 ml) with homogenization buffer (×1) and PBS/0.1% (v/v) Tween 20 (×2) for 10 min at 4° before further processing.

Phosphatase treatment. After treatment of HL-60/Bcl-2 cells with bryostatin 1, staurosporine, or UCN-01, cell lysates were incubated in the presence of 1.5 units/ml of alkaline phosphatase (Sigma) for 4 hr at 37°. SDS-PAGE immunoblot analysis was then performed using anti-Bcl-2 monoclonal antibody and enhanced chemiluminescence-based detection as described above. Alternatively, immunoprecipitates containing ^{32}P-labeled Bcl-2 protein were washed (×3, 1 ml) each for 5 min at 4° in "phosphatase buffer" [25 mM Tris·HCl, pH 7.5, 37°, 2 mM $MgCl_2$, 0.01% (v/v) Tween 20]. Beads were resuspended in 50 μl of the same buffer and incubated (60 min, 37°) with or without PP2A (10 munits/ml final concentration) and in the presence or absence of the specific protein serine/threonine phosphatase inhibitor OA (0.5 mM). Reactions were quenched by the addition of 10 μl of a 5× SDS-PAGE sample buffer followed by boiling (10 min) and subjected to SDS-PAGE using a 12% gel. Separated proteins were transferred to nitrocellulose, followed by both autoradiography to visualize ^{32}P-labeled proteins and Western immunoblotting to detect Bcl-2 protein.

Bcl-2/Bax immunoprecipitation studies. After drug treatment, cells were lysed, and Bcl-2 immunoprecipitations was performed as described in the preceding section. Equal quantities of protein (200 μg/condition) were immunoprecipitated with Bcl-2 monoclonal antibody, after which immunoconjugates were transferred to nitrocellulose and Western blot analysis was performed using Bax polyclonal antibodies.

Statistical analysis. The significance of differences between experimental conditions was determined using the Student's *t* test for unpaired observations.

Results

Western analysis revealed that HL-60/Bcl-2 cells exhibited a ≈5-fold increase in Bcl-2 expression compared with cells containing empty-vector (pCEP4) or untransfected controls (Fig. 1). In contrast, levels of Bax, Bcl-x_L, and Mcl-1 were equivalent in HL-60/Bcl-2 and pCEP4 cells. The proapoptotic protein Bcl-x_s was undetectable in any of these cell lines under basal conditions (not shown).

When cells were exposed to varying concentrations of ara-C for 6 hr, DNA fragmentation was significantly reduced in HL-60/Bcl-2 cells compared with HL-60/pCEP4 (Fig. 2A). A parallel reduction in the percentage of apoptotic cells was also observed in the Bcl-2-overexpressing line (Fig. 2B). Differences were most pronounced in cells exposed to ara-C at concentrations of 5–100 μM. Similarly, qualitative results of agarose gel electrophoresis revealed decreased intensity of ethidium bromide-stained bands corresponding to oligonucleosomal DNA fragments (data not shown). Thus, increased expression of Bcl-2 partially protected HL-60 cells from the DNA-damaging actions of ara-C. In this and in subsequent studies, responses of untransfected HL-60 cells were essentially equivalent to those of empty-vector (pCEP4) controls (data not shown).

Overexpression of Bcl-2 has been reported to protect HeLa cells from the early induction of apoptosis by certain chemotherapeutic drugs (e.g., aphidicolin) without preservation of clonogenic potential (19). Therefore, the effects of increased Bcl-2 expression were examined in relation to the clonogenic survival of HL-60 cells exposed to ara-C (Fig. 3). As noted in the case of DNA damage, Bcl-2-mediated protection from the lethal effects of ara-C was incomplete. Nevertheless, significant increases in survival were noted in Bcl-2-overexpressing

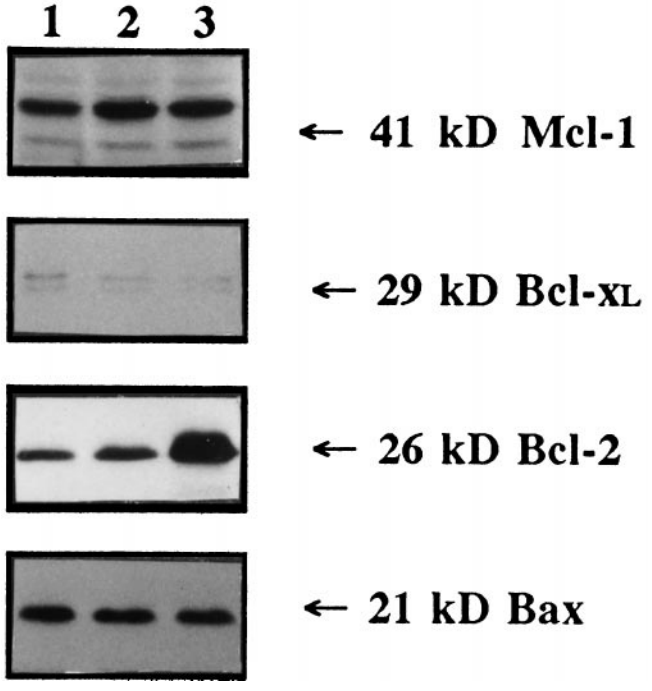

Fig. 1. Western analysis was performed on protein (25 μg) isolated from the untransfected parental line, HL-60 cells transfected with the pCEP4 vector alone, and cells stably transfected with a vector containing the Bcl-2 coding region under the control of a CMV promoter. After electrophoresis and transfer, blots were probed with primary antibody to Bcl-2, Bcl-x_L, Bax, and Mcl-1; exposed to horseradish peroxidase-conjugated secondary antibody; and developed using enhanced chemiluminescence reagents as described in the text. *Lane 1*, parental HL-60 cells. *Lane 2*, HL-60/pCEP4. *Lane 3*, HL-60/Bcl-2. A representative blot is shown; two others yielded equivalent results.

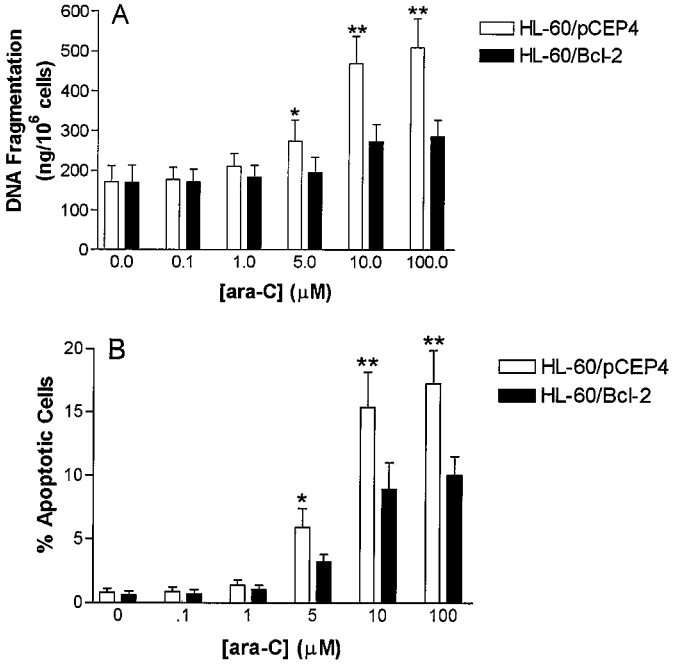

Fig. 2. HL-60/pCEP4 and HL-60/Bcl-2 cells were incubated with the indicated concentrations of ara-C for 6 hr, after which the amount of low-molecular-weight DNA fragmentation (A) and percentage of apoptotic cells (B) monitored as described in Materials and Methods. Values represent the means for three separate experiments performed in triplicate ± standard deviation ∗, Significantly greater than values for HL-60/Bcl-2 ($p \leq 0.05$). ∗∗, $p \leq 0.01$.

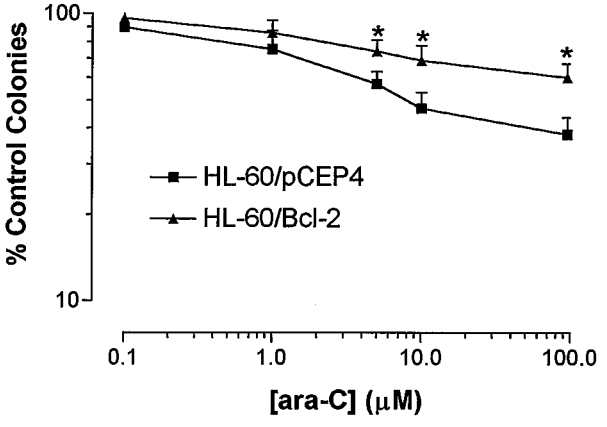

Fig. 3. HL-60/pCEP4 and HL-60/Bcl-2 cells were incubated with the indicated concentration of ara-C for 6 hr, washed thoroughly in fresh medium to remove all drug, and plated in soft agar as described in the text. At the end of 12-dayincubation, colonies, consisting of groups of ≥50 cells, were scored with the aid of an inverted microscope, and colony formation for each condition was expressed in relation to untreated controls. The cloning efficiency of control cells was ≈40%. Values represent the mean ± standard deviation for four separate experiments performed in triplicate. ∗, Significantly greater than values for HL-60/pCEP4 ($p \leq 0.05$).

cells at ara-C concentrations ≥5 μM. Moreover, clonogenic HL-60/Bcl-2 cells exhibited a 4-fold increase in ara-C IC_{50} values compared with their pCEP4 counterparts (data not shown). In separate studies, the S-phase fraction of HL-60/Bcl-2 and HL-60/pCEP4 cells was found to be equivalent (40 ± 2% versus 41 ± 3%; $p \geq 0.05$; data not shown), indicating that reduced sensitivity of Bcl-2-overexpressors did not stem from cytokinetic factors. Collectively, these findings document that overexpression of the Bcl-2 protein confers resistance to ara-C-mediated lethality in this cell line.

Previous studies have shown that chronic exposure of HL-60 cells to bryostatin 1 potentiates ara-C-related apoptosis (11, 12), as does a more acute exposure to the potent but nonspecific PKC inhibitor staurosporine (15). Accordingly, an attempt was made to determine whether these agents exerted similar effects in cells overexpressing Bcl-2. Cells were also exposed to UCN-01, a more specific inhibitor of PKC than staurosporine that, in contrast to the latter agent, exhibits *in vivo* antitumor activity (20). As noted above, a 6-hr exposure to 10 μM ara-C was less effective in inducing apoptosis in HL-60/Bcl-2 cells than in their empty-vector counterparts (Fig. 4A). However, when cells were preincubated with bryostatin 1 (10 nM; 24 hr), an exposure that was ineffective by itself, a significant increase (≈100%) in ara-C-related apoptosis was noted in both cell lines. Although Bcl-2 overexpression continued to afford some protection from the combined effects of bryostatin 1 and ara-C, HL-60/Bcl-2 cells treated with both agents exhibited levels of apoptosis that were statistically indistinguishable from those observed in HL-60/pCEP4 cells exposed to ara-C alone ($p \geq 0.05$). Comparable patterns were seen with both staurosporine and UCN-01. Thus, incubation of HL-60/Bcl-2 cells with 50 nM staurosporine or 300 nM UCN-01 (7 hr each) alone was ineffective in inducing apoptosis but significantly increased the degree of ara-C-mediated cell death. Specifically, a significantly higher percentage of HL-60/Bcl-2 cells exposed to both staurosporine and ara-C exhibited apoptosis than HL-60/pCEP4 cells treated with ara-C alone ($p \leq 0.05$), whereas incubation of overexpressing cells with UCN-01 and ara-C led to equivalent degrees of cell death ($p \geq 0.05$). No effects were noted when cells were exposed to HA-1004, a moderately selective inhibitor of PKA (data not shown). Coadministration of bryostatin 1 with UCN-01 led to only a modest further increase in ara-C-mediated apoptosis; nevertheless, levels exceeded those observed in ara-C-treated HL-60/pCEP4 cells ($p \leq 0.01$). Parallel results were obtained when DNA fragmentation was monitored in the HL-60/Bcl-2 line (Fig. 4B). In these cells, ara-C treatment alone induced barely discernible DNA fragmentation, and bryostatin 1, staurosporine, and UCN-01 were ineffective individually. However, combined treatment resulted in the unequivocal induction of DNA degradation. Essentially equivalent results were obtained in a second Bcl-2-overexpressing clone (HL-60/Bcl-2-B5; data not shown). Together, these findings demonstrate that bryostatin 1, staurosporine, and UCN-01 are each capable of overcoming, at least in part, resistance to ara-C-related apoptosis conferred by Bcl-2 overexpression.

Because HL-60/Bcl-2 cells remained slightly less susceptible to the apoptotic actions of ara-C and bryostatin 1 than their empty-vector counterparts, effects on PKC down-regulation were compared in the two cells (Fig. 5A). Both cell lines exhibited a dose-dependent reduction in total cellular PKC

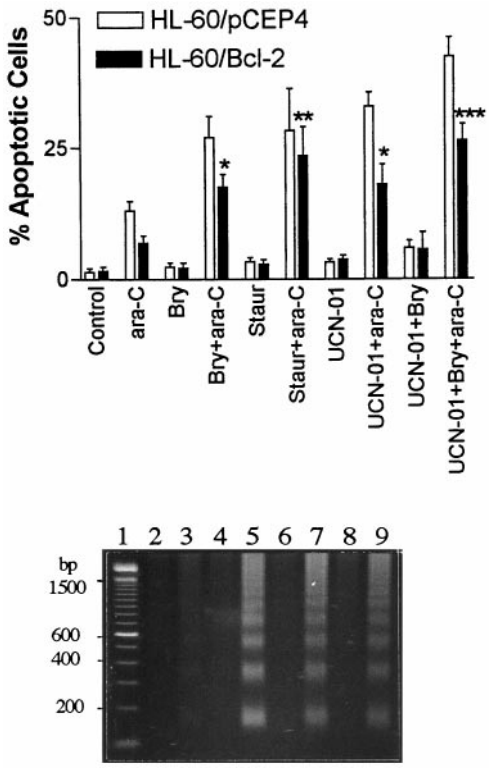

Fig. 4. *Top,* HL-60/pCEP4 and HL-60/Bcl-2 cells were preincubated with 10 nM bryostatin 1 (*Bry*) for 24 hr before a 6-hr exposure to 10 μM ara-C. Alternatively, cells were incubated with 50 nM staurosporine (*Staur*) or 300 nM UCN-01 for 1 hr, followed by an identical ara-C treatment. At the end of this period, the percentage of apoptotic cells was determined as described in the text. Values represent the mean ± standard deviation for three separate experiments performed in triplicate. *, Values equivalent to those for pCEP4 cells treated with ara-C alone ($p \geq 0.05$). **, Values greater than those for ara-C-treated pCEP4 cells ($p \leq 0.5$). ***, $p \leq 0.01$. *Bottom*, after treatment as described above, DNA was extracted from HL-60/Bcl-2 cells, subjected to agarose gel electrophoresis using ethidium bromide-impregnated gels, and photographed under UV light. *Lane 1*, DNA ladder. *Lane 2*, control. *Lane 3*, ara-C. *Lane 4*, staurosporine. *Lane 5*, staurosporine plus ara-C. *Lane 6*, UCN-01. *Lane 7*, UCN-01 plus ara-C. *Lane 8*, bryostatin 1. *Lane 9*, bryostatin 1 plus ara-C. Two additional studies yielded equivalent results.

activity after a 24-hr exposure to bryostatin 1; moreover, the degree of PKC down-regulation was equivalent in the two cell types. In view of evidence that bryostatin 1-induced potentiation of ara-C-related apoptosis is decreased under conditions in which cellular maturation occurs (21), differentiation induction was also monitored (Fig. 5B). Neither HL-60/Bcl-2 nor pCEP4 cells exhibited a significant increase in plastic adherence after a 3-day exposure to 10 nM bryostatin 1, whereas an equivalent exposure to PMA induced adherence in the large majority of cells. Similar results were obtained when CD11b expression was monitored (data not shown). Consequently, the modestly reduced apoptotic response of HL-60/Bcl-2 cells to bryostatin 1 and ara-C could not be attributed to differentiation induction by bryostatin 1 or failure to down-regulate PKC activity.

It has been shown that overexpression of Bcl-2 by leukemic cells inhibits ara-C- and taxol-induced cleavage of the Yama protease (caspase-3) and degradation of one of its substrates (PARP) (7). Consequently, the effects of combining ara-C with bryostatin 1, staurosporine, or UCN-01 in HL-60/Bcl-2

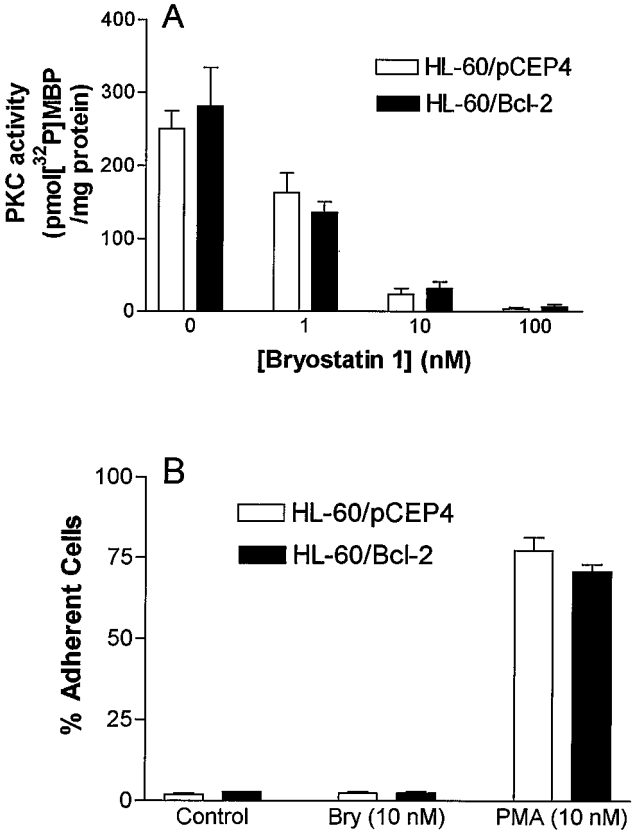

Fig. 5. HL-60/pCEP4 and HL-60/Bcl-2 cells were exposed to the designated concentration of bryostatin 1 for 24 hr, after which cells were lysed and total cellular PKC activity was determined by monitoring phosphorylation of myelin basic protein as described in the text. A, Values represent the mean ± standard deviation for three separate experiments performed in triplicate. B, Percentage of adherent cells was determined after a 72 hr exposure to 10 nM bryostatin 1 or PMA. Values represent the mean ± standard deviation for three separate studies performed in triplicate.

cells were examined in relation to activation of the apoptotic protease cascade (Fig. 6). Consistent with previous reports (6, 7), ara-C treatment increased caspase-3 activity and reduced levels of the32-kDa Yama protein in HL-60/pCEP4 cells but exerted marginal effects in their Bcl-2-overexpressing counterparts (Fig. 6, A and B). Correspondingly, PARP was cleaved from its native 115-kDa form to an 85-kDa degradation product in HL-60/pCEP4 but not in HL-60/Bcl-2 cells (Fig. 6C). Bryostatin 1, staurosporine, or UCN-01 administered alone failed to increase caspase-3 activity, reduce levels of the 32-kDa species or induce PARP cleavage in HL-60/Bcl-2 cells. However, coadministration of each of these agents with ara-C resulted in a significant increase in caspase-3 activity, a reduction in expression of the 32-kDa species, and PARP degradation in the Bcl-2-overexpressing cell line. Thus, bryostatin 1, staurosporine, or UCN-01 each restored the ability of ara-C to activate the apoptotic protease cascade in cells overexpressing Bcl-2

Parallel effects were observed when clonogenic survival was monitored (Fig. 7). Specifically, the capacity of Bcl-2 to protect clonogenic cells from ara-C-mediated cytotoxicity was abrogated by coadministration of concentrations of bryostatin 1, staurosporine, and UCN-01 that were minimally effective alone. In each case, clonogenicity was reduced to levels equivalent to those observed in empty-vector controls exposed to ara-C.

We reported recently that treatment with bryostatin 1 fails to down-regulate Bcl-2 expression in HL-60 cells, although it does lead to a broadening of the Bcl-2 protein band, presumably reflecting gel retardation accompanying phosphorylation (18). This event has been widely reported to occur in cells exposed to taxol and other microtubule-active agents in association with induction of apoptosis (22), and it has been suggested that phosphorylation antagonizes the protective capacity of Bcl-2 in this setting (23). Studies were therefore undertaken to determine whether bryostatin 1, staurosporine, and UCN-01 exerted a comparable effect in the Bcl-2-overexpressing cell line (Fig. 8). Exposure of cells to taxol (1 μM; 24 hr) resulted in a modest broadening of the Bcl-2 band and the appearance of a distinct, slowly migrating species, which was consistent with previously reported findings (24) (Fig. 8, *top*). Treatment of cells with ara-C resulted in a very slight widening of the band but did not result in resolution of a distinct species. However, when cells were exposed to staurosporine, UCN-01, and (particularly) bryostatin 1, there was a marked broadening of the Bcl-2 band, with a portion of the slow-mobility protein comigrating with a putative phosphorylated species seen in cells treated with taxol. Coadministration of ara-C resulted in either no significant change (UCN-01 or staurosporine) or a slight further broadening of the Bcl-2 band (bryostatin 1). Repeat probing of the membranes with antibodies directed against tubulin confirmed equivalent loading of lanes for each condition. Coincubation with cycloheximide (10 μg/ml) failed to abrogate the bryostatin 1 effect or that seen with each of the other agents (data not shown). Last, treatment of cell lysates with alkaline phosphatase before Western analysis eliminated the observed broadening and altered mobility of the Bcl-2 protein bands (Fig. 8, *bottom*), further implicating phosphorylation in this phenomenon.

To determine more rigorously whether bryostatin 1, staurosporine, and UCN-01 did in fact modify Bcl-2 phosphorylation in HL-60/Bcl-2 cells, *in vivo* metabolic labeling with $^{32}PO_4$-orthophosphate and ^{35}S-methionine was performed (Fig. 9). In these experiments, prelabeled cells were incubated with the indicated agent, after which immunoprecipitated Bcl-2 protein was monitored by autoradiography. Treatment with bryostatin 1, staurosporine, or UCN-01 failed to increase ^{35}S-methionine incorporation (Fig. 9A), demonstrating that drug exposure did not alter Bcl-2 protein synthesis. In contrast, a clear increase in$^{32}PO_4$-orthophosphate radiolabeling was noted in immunoprecipitated protein isolated from drug-treated cells (Fig. 9B), a phenomenon similar to that previously reported in cells exposed to taxol (22). Phosphorylation of Bcl-2 protein by each of the agents was reduced after treatment of lysates with PP2A; the latter effect was abrogated by the phosphatase inhibitor OA. Together, these findings indicate that bryostatin 1 and the PKC inhibitors staurosporine and UCN-01 induce phosphorylation of the Bcl-2 protein, raising the possibility that this action may contribute to potentiation of ara-C-related apoptosis in Bcl-2-overexpressing leukemic cells.

In view of evidence implicating Bax as a key mediator of apoptosis (2), expression of Bax and its interaction with Bcl-2 were examined in drug-treated cells (Fig. 10). In contrast to results shown above (Fig. 8), the mobility of the Bax protein

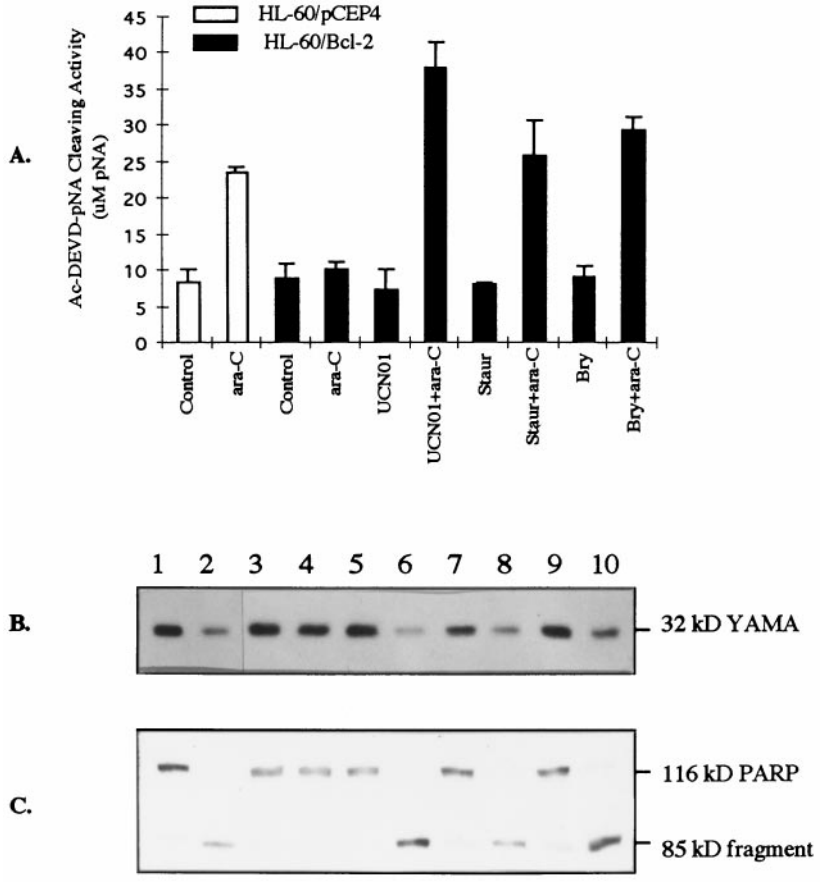

Fig. 6. HL-60/Bcl-2 cells were exposed to bryostatin 1 (*Bry*; 10 nM; 24 hr), staurosporine (*Staur*; 50 nM; 1 hr), or UCN-01 (300 nM; 1 hr) before a 6-hr incubation with 10 μM ara-C, after which CPP32 activity was monitored using an enzymatic method (A). Values, expressed as the concentration of the Ac-DEVD-*p*-nitroanilide substrate cleaved over a 1-hr interval, represent the mean $\pm$ 1 standard deviation for triplicate determinations; two additional experiments yielded equivalent results. Alternatively, protein (10 μg) was extracted from cell lysates and subjected to Western analysis using antibodies to CPP32 (B) or PARP (C) as described in the text. *Lanes 1 and 2*, characteristic responses using protein isolated from control and ara-C-treated HL-60/pCEP4 cells. Two additional experiments yielded equivalent results.

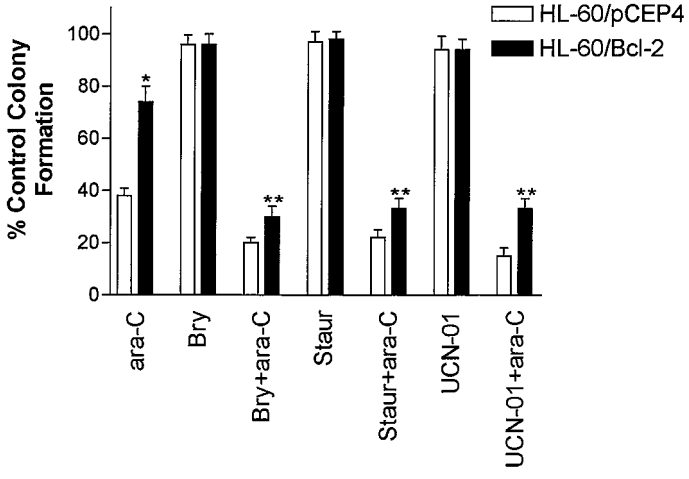

Fig. 7. HL-60/pCEP4 and HL-60/Bcl-2 cells were preincubated with (a) bryostatin 1 (*Bry*; 10 nM; 24 hr), (b) staurosporine (*Staur*; 50 nM; 1 hr), or (c) UCN-01 (300 nM; 1 hr), followed by 10 μM ara-C for an additional 6 hr. The cells were washed thoroughly to remove all drugs and plated in soft agar as described in the text. At the end of 12 days, colonies were scored and survival expressed in relation to control cell growth (cloning efficiency, $\approx$40%). Values represent the mean $\pm$ standard deviation for three separate experiments performed in triplicate. *, Survival greater than that for pCEP4 cells exposed to ara-C alone ($p < 0.05$). **, Survival equivalent to that for pCEP4 cells exposed to ara-C alone ($p > 0.05$).

band was unperturbed by treatment with bryostatin 1, staurosporine, or UCN-01 with or without ara-C (*10A*). Moreover, when immunoprecipitated Bcl-2 protein was probed with anti-Bax antibody, no reduction in coimmunoprecipitating

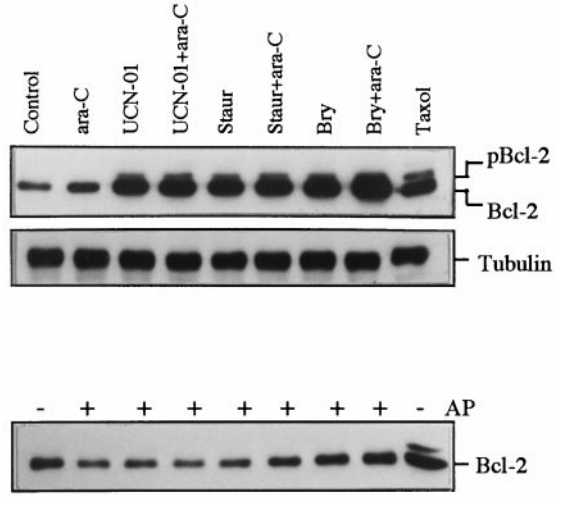

Fig. 8. *Top*, HL-60/Bcl-2 cells were exposed to ara-C (10 μM; 6 hr), bryostatin 1 (*Byr*; 10 nM; 24 hr), staurosporine (*Staur*; 50 nM; 7 hr), UCN-01 (300 nM; 7 hr), or the combination of each of these agents and ara-C, after which protein was extracted (10 μg) and Bcl-2 expression was assessed by Western analysis as described in the text. *Last lane*, contains protein from cells exposed to taxol (1 μM; 24 hr). *Middle*, membranes were stripped and reprobed with antibodies directed against tubulin). *Bottom*, after treatment of cells as described above, expression of Bcl-2 was examined by Western analysis after incubation of protein extracts with (+) or without (−) 1.5 units/ml of alkaline phosphatase (*AP*) for 4 hr at 37°. *Lanes*, correspond to the conditions shown at the *top*. Untreated extracts from taxol-treated cells are shown to illustrate the position of the slowly migrating Bcl-2 species (*last lane*). A representative study is shown; two separate experiments yielded equivalent results.

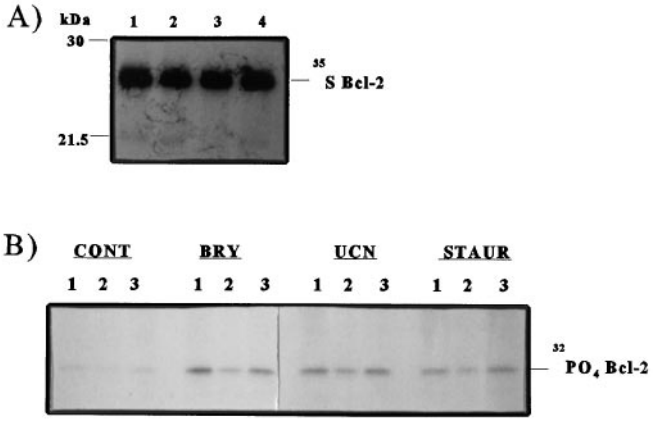

Fig. 9. A, HL-60/Bcl-2 cells were prelabeled with ^{35}S-methionine as described in the text and exposed to bryostatin 1 (10 nM; 24 hr), UCN-01 (300 nM; 7 hr), or staurosporine (50 nM; 7 hr) as above. Equal quantities of Bcl-2 protein were immunoprecipitated, separated by SDS-PAGE, and visualized by autoradiography as described in the text. *Lane 1*, control. *Lane 2*, bryostatin 1. *Lane 3*, UCN-01. *Lane 4*, staurosporine. B, HL-60/Bcl-2 cells were prelabeled with ^{32}P-orthophosphate and treated as described above, and equal amounts of protein (100 μg) were subjected to electrophoresis before autoradiography. For each condition: *lane 1*, untreated immunoprecipitated Bcl-2; *lane 2*, immunoprecipitated Bcl-2 treated with PP2A (10 munits/ml); *lane 3*, immunoprecipitated Bcl-2 treated with PP2A plus 0.5 mM OA. *CONT*, control; *BRY*, bryostatin 1; *STAUR*, staurosporine.

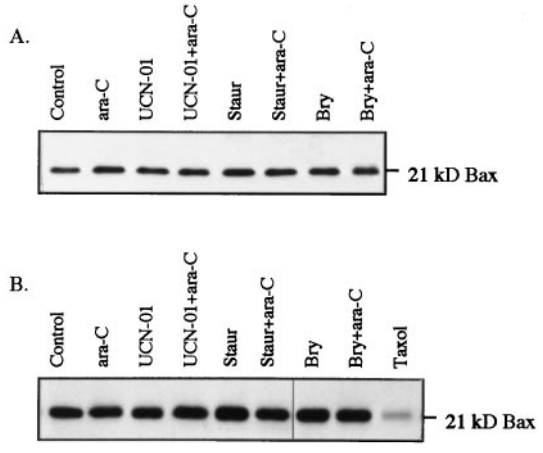

Fig. 10. A, After treatment with ara-C with or without bryostatin 1 (*Bry*), staurosporine (*Staur*), or UCN-01 as above, cells were lysed, and Bax protein (15 μg/condition) was evaluated by Western analysis using anti-Bax monoclonal antibodies and enhanced chemiluminescence reagents as described in Materials and Methods. B, Alternatively, equal quantities of Bcl-2 protein (200 μg/condition) were immunoprecipitated as above and separated by SDS-PAGE, and coimmunoprecipitating Bax was determined by Western analysis. Extracts from cells exposed to 1 μM taxol for 24 hr are shown for comparison (*last lane*). A representative experiment is shown; two additional studies yielded equivalent results.

Bax was observed in material obtained from drug-treated cells (Fig. 10B). In contrast, a clear reduction in coimmunoprecipitating Bax was noted in cells treated with taxol. This finding is consistent with the results of an earlier report in which altered Bcl-2 mobility after taxol treatment of prostate cancer cells was associated with reduced binding to Bax (24). These observations suggest that the functional consequences of Bcl-2 phosphorylation by bryostatin 1, staurosporine, and UCN-01 are distinct, at least in some respects, from those associated with taxol.

Discussion

Our results demonstrate that protection from ara-C-induced apoptosis conferred by overexpression of Bcl-2 can be substantially circumvented by the PKC inhibitors staurosporine and UCN-01, as well as by the PKC activator bryostatin 1. Previous studies have shown that ectopic expression of Bcl-2 protects leukemic cells from ara-C (6, 7) and that this capacity results from interference with a distal step in the cell death pathway rather than from cytokinetic or biochemical perturbations (25). Although the mechanism underlying this phenomenon remains to be elucidated, recent evidence suggests that Bcl-2 antagonizes the lethal effects of reactive oxygen intermediates generated by ara-C exposure (26). Such findings, along with the observations that leukemic blasts expressing high levels of Bcl-2 protein (a) tend to be resistant to apoptosis after *ex vivo* exposure to ara-C (8) and (b) correlate with poor responses to chemotherapy (5), provide a compelling rationale for developing strategies capable of overcoming Bcl-2-mediated cytoprotective effects.

The current study was prompted by evidence that chronic exposure of cells to bryostatin 1, which induces extensive PKC down-regulation, potentiates ara-C-related apoptosis in HL-60 cells in a dose- and time-dependent manner (12). Similarly, PKC inhibitors such as staurosporine that directly inhibit enzyme activity exert similar effects (15). There is substantial evidence that PKC acts to oppose apoptotic events. For example, the tumor-promoting phorbol diester PMA antagonizes growth factor deprivation-induced apoptosis in hematopoietic cells (13). In addition, elevations in PKC activity by endogenous or exogenous diglycerides or acute exposures to pharmacological PKC activators oppose ceramide-mediated apoptosis in human myeloid leukemia cells (27). Conversely, PKC inhibitors have been shown to induce apoptosis in a wide variety of cell types (14). Based on these and related findings, it has been proposed that alterations in PKC activity shift the balance between downstream signaling elements associated with cell survival (e.g., mitogen-activated protein kinases) or cell death (e.g., stress-activated protein kinases) (28). The results of the current study (e.g., Figs. 4, 6, and 7) indicate that agents that down-regulate or inhibit PKC are capable of potentiating drug-induced apoptosis in cells overexpressing Bcl-2. Nevertheless, Bcl-2 overexpressors remained less sensitive to the drug combinations than their empty-vector counterparts, suggesting that reversal of Bcl-2-mediated cytoprotective effects by this strategy is not complete. It is interesting that staurosporine and UCN-01 were able to restore ara-C sensitivity in Bcl-2 overexpressors, inasmuch as (a) PKC inhibitor-induced apoptosis (particularly that elicited by staurosporine) is known to be inhibitable by Bcl-2 (10, 29) and (b) staurosporine, when administered at high concentrations (e.g., 1 μM) is a potent inducer of apoptosis by itself (14). In this regard, we reported previously that administration of subtoxic concentrations of staurosporine (e.g., 50 nM) potentiates ara-C-induced apoptosis in parental HL-60 cells (15). One possible explanation for this phenomenon is that the capacity of these agents to induce apoptosis directly, most notably at high drug concentrations, involves inhibition of kinases other than PKC; in contrast, facilitation of ara-C-related apoptosis may be primarily PKC dependent. In this context, staurosporine-mediated apoptosis in murine mammary carcinoma cells (FT210)

has been attributed to dephosphorylation and inappropriate activation of the $p34^{cdc2}$ kinase (30), and similar findings have been reported in Jurkat cells undergoing apoptosis in response to UCN-01 (20). Additional studies will be required to resolve this issue.

Considerable attention has focused on activation of the protease cascade in drug-induced and other forms of apoptosis, as well as on the ability of Bcl-2 and related proteins to inhibit this process. For example, increased expression of Bcl-2 and Bcl-x_L has been shown to block staurosporine-induced activation of CPP32 and accompanying PARP cleavage in Jurkat cells but not that induced by Fas/APO-1 (10), although the latter finding has not been universally observed (31). Together, these results indicate that in the case of some, but perhaps not all, cytotoxic stimuli, Bcl-2 functions upstream of a critical cysteine protease involved in the degradation phase of apoptosis. The current findings, in agreement with recent reports (6, 7), demonstrate that activation of the protease cascade in human leukemia cells by ara-C is also blocked by increased Bcl-2 expression. Significantly, our results show that blockade of protease activation associated with overexpression of Bcl-2 can be substantially overcome by the administration of otherwise subeffective concentrations of bryostatin 1, staurosporine, or UCN-01. It is possible that these agents act directly to interfere with Bcl-2 function (see below); alternatively, they may act in an as-yet-undetermined way to increase the ability of ara-C to modulate intracellular concentrations of lipid effector molecules (32) or reactive oxygen intermediates (26). It is also conceivable that interruption of the PKC pathway lowers the threshold for caspase-3 activation, although to the best of our knowledge, a connection between these events has not been established. In this regard, it has recently been demonstrated that Bcl-2 inhibits mitochondrial release of cytochrome *c*, which has been implicated in activation of caspase-3 (33, 34). Accordingly, efforts are under way to determine whether Bcl-2 phosphorylation induced by bryostatin 1, staurosporine, or UCN-01 is associated with redistribution of cytochrome *c* from mitochondria to cytosol.

The observation that bryostatin 1, staurosporine, and UCN-01 induce phosphorylation of the Bcl-2 protein in association with potentiation of ara-C-related apoptosis is noteworthy, particularly in view of recent reports linking certain forms of drug-induced apoptosis to this process. For example, exposure of cells to various agents that disrupt microtubular assembly/disassembly (e.g., vincristine, taxol, taxotere) leads to the appearance of a distinct, phosphorylated Bcl-2 species exhibiting decreased mobility on gel electrophoresis; moreover, this event involves Raf-1 activation (23). In contrast, antimetabolites have not been reported to alter Bcl-2 phosphorylation status in this manner. Although the mechanism by which phosphorylation of Bcl-2 promotes apoptosis remains to be determined, studies in prostate cancer cells have shown that taxol-induced phosphorylation of Bcl-2 reduces heterodimerization with the proapoptotic protein Bax (24), a process that has previously been reported to oppose cell death (2). The results described herein demonstrate that a similar phenomenon occurs in HL-60 leukemic cells exposed to taxol. However, in view of the data shown in Fig. 10, which failed to demonstrate a decrease in Bcl-2/Bax heterodimerization, an alternative mechanism seems to be operative in leukemic cells treated with bryostatin 1, staurosporine, and UCN-01. In this context, it has been shown recently that mutant forms of Bcl-2 and Bcl-x_L lacking a domain containing the major phosphorylation sites exhibit increased antiapoptotic activity compared with the full-length Bcl-2 protein but do not display altered binding to Bax (35). Taken in conjunction with evidence that Bcl-x_L mutants impaired in their ability to heterodimerize with Bax retain most of their capacity to inhibit apoptosis (36), it is likely that cell death-regulatory mechanisms exist that do not involve Bax or, alternatively, binding of Bcl-2 to this protein. It may also be relevant that in contrast to results obtained with taxol (23), bryostatin 1 and PKC inhibitors resulted in a diffuse broadening of the Bcl-2 band, an event that was insensitive to cycloheximide. These phenomena could reflect specific patterns of Bcl-2 phosphorylation, the functional consequences of which may be unrelated to Bax.

It is important to note that although bryostatin 1-induced Bcl-2 phosphorylation was associated with increased susceptibility of HL-60/Bcl-2 cells to ara-C-induced apoptosis, an earlier study demonstrated that phosphorylation of Bcl-2 by bryostatin 1 rendered 32D cells less sensitive to growth factor deprivation-induced cell death (16). This discrepancy could stem from differential responses of normal versus neoplastic hematopoietic cells to bryostatin 1; alternatively, it could reflect site-specific phosphorylation effects. On the other hand, the current results, particularly those pertaining to bryostatin 1, are consistent with previous reports involving Jurkat cells stably transfected with v-Ha-*ras* (17, 37). In these studies, down-regulation of PKC by chronic exposure to PMA led to both phosphorylation of Bcl-2 and induction of apoptosis. The ability of UCN-01 and staurosporine to exert a similar effect in HL-60 cells suggests that agents that down-regulate or inhibit PKC may share a common mechanism of action with respect to modulation of Bcl-2 phosphorylation. It is nevertheless interesting that in Jurkat cells constitutively expressing activated $p21^{ras}$, staurosporine was found to block PMA-induced Bcl-2 phosphorylation but not apoptosis (37). Whether this phenomenon is cell type specific or unique to $p21^{ras}$-related apoptosis remains to be determined. Finally, the functional significance of post-translational modification of Bcl-2 and related proteins in the regulation of apoptosis has been underscored by two recent reports demonstrating that phosphorylation of the proapoptotic Bcl-2 family member BAD antagonizes both heterodimerization with Bcl-x_L and induction of cell death (38, 39). Conversely, a model has been proposed in which Bcl-2 phosphorylation lowers the threshold for cell death, whereas dephosphorylation has the net effect of promoting cell survival (40). According to this hypothetical model, the functional status of Bcl-2 may ultimately depend on the relative activities of kinases and phosphatases putatively responsible for phosphorylation and dephosphorylation of the protein, respectively. Thus, sustained PKC activity may be required for constitutive activation of phosphatases that maintain Bcl-2 in its fully functional dephosphorylated state. To the extent that PKC regulates the balance between kinase and phosphatase activity, reduction of PKC activity (e.g., by down-regulation, as in the case of bryostatin 1, or direct inhibition, in the case of UCN-01 or staurosporine) could shift the balance toward Bcl-2 phosphorylation and, consequently, cell death. Studies designed to test this hypothesis are in progress.

In summary, the novel findings of this report include the following: (a) agents known to inhibit or down-regulate PKC restore the ability of ara-C to induce apoptosis in otherwise resistant Bcl-2-overexpressing leukemic cells; (b) this phenomenon is accompanied by a corresponding reduction in leukemic cell clonogenic capacity; (c) subtoxic concentrations of bryostatin 1, staurosporine, or UCN-01 circumvent Bcl-2-associated blockade of ara-C-mediated apoptotic protease activation; and (d) these events are accompanied by Bcl-2 phosphorylation but not by a reduction in Bcl-2/Bax heterodimerization. Collectively, these findings raise the possibility that agents targeting signaling pathways involved in the regulation of Bcl-2 function may overcome drug resistance resulting from interruption of a distal step in the cell death pathway. In view of accumulating evidence that Bcl-2 may be an important determinant of clinical response in leukemia (5), further efforts to explore this therapeutic strategy appear warranted.

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