Protein Kinase C-dependent Activation of the Tumor Necrosis Factor Receptor-mediated Extrinsic Cell Death Pathway Underlies Enhanced Apoptosis in Human Myeloid Leukemia Cells Exposed to Bryostatin 1 and Flavopiridol1

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Abstract

Interactions between the protein kinase C activator bryostatin 1 and the cyclin-dependent kinase inhibitor flavopiridol (FP) have been examined in human myeloid leukemia cells (U937 and HL-60). Previous studies have demonstrated synergistic induction of apoptosis in leukemia cells exposed to the potent differentiation-inducer phorbol 12-myristate 13-acetate (PMA) in conjunction with FP [L. Cartee et al., Cancer Res., 61: 2583–2591, 2001]. Although bryostatin 1 (10 nM) is a very weak inducer of differentiation compared with PMA in these cells, coadministration of a minimally toxic concentration of FP (100 nM) did not promote bryostatin 1-related maturation but instead caused a marked increase in mitochondrial damage (e.g., cytochrome c release; loss of ΔΨm), caspase activation, poly(ADP-ribose) polymerase cleavage, and apoptosis. Bryostatin 1/FP-induced apoptosis was significantly diminished in cells ectopically expressing dominant-negative Fas-associated death domain or by coadministration of tumor necrosis factor (TNF)-α soluble receptors, implicating the extrinsic pathway in bryostatin 1/FP actions. Enhanced apoptosis in bryostatin 1/FP-treated cells was accompanied by down-regulation of Mcl-1 and a sustained increase in TNF-α release. The selective protein kinase C inhibitor GFX blocked TNF-α and cytochrome c release in bryostatin 1/FP-treated cells and attenuated apoptosis.

Finally, coadministration of bryostatin 1 (or PMA) with FP induced a marked increase in apoptosis in U937 cells ectopically expressing an NH2-terminal phosphorylation loop-deleted Bcl-2 protein, which are otherwise highly resistant to FP-mediated lethality. Taken together, these findings suggest that synergistic induction of apoptosis by bryostatin 1 and FP does not stem from disruption of the leukemic cell maturation process but instead results from enhanced release of TNF-α and activation of the extrinsic apoptotic cascade, culminating in cell death.

Introduction

Apoptosis is a highly conserved process of cell suicide that involves the activation of a family of cysteine proteinases referred to as caspases (1). Apoptosis proceeds via two distinct biochemical caspase cascades, designated as the intrinsic or extrinsic pathways (2, 3). The intrinsic or mitochondrial pathway is triggered by ionizing radiation or cytotoxic drugs and is initiated by cyt c release from the mitochondria (4). When cyt c is released into the cytoplasm, it forms a multimeric protein complex with apoptosis activating factor-1 and procaspase-9 referred to as the apoptosome. The apoptosome then cleaves and activates downstream effector caspases such as caspase-3, caspase-6, and caspase-7, which promote cell death by initiating DNA fragmentation and intracellular protein degradation (5). In contrast, the extrinsic apoptotic pathway is receptor mediated and involves the recruitment of procaspase-8 to the DISC of cell surface death receptors (6, 7). The DISC contains the FADD, an adapter protein with a death domain effector sequence that binds to a homologous sequence within procaspase-8. After ligand binding and recruitment by FADD, procaspase-8 oligomerization triggers its autoactivation by self-cleavage. Caspase-8 then activates the downstream effector caspases via type I or type II receptor-mediated pathways (8, 9).

1 The abbreviations used are: cyt c, cytochrome c; BisM, bisindolylmaleimide; BD-FMK, BOC-Asp(OMe)-fluoromethylketone; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; CHX, cycloheximide; CI, combination index; DIOc, 3,3-dihexyloxacarbocyanine; DISC, death-inducing signaling complex; DN, dominant-negative; FADD, Fas-associated death domain; FP, flavopiridol; MEK, MAP/ERK kinase; PARP, poly(ADP-ribose)polymerase; PI, propidium iodide; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TBS-T, Tris-buffered saline with 0.1% Tween 20; TNF-α, tumor necrosis factor α; TNFR, TNF receptor; TNFSR, TNF soluble receptor; VP16, etoposide; pRb, retinoblastoma protein.

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FP is a CDKI that interacts with the adenine-binding pocket of CDKs at concentrations ~100 nM for CDKs 1, 2, 4, and 6, and 300 nM for CDK 7, the CDK-activating kinase (10). FP has also been found to inhibit the CDK9/T1 transcription elongation factor b complex and thereby act as a transcriptional repressor (11). FP induces either G1 and/or G2-M cell cycle-arrest (12) and is an effective inducer of apoptosis in human leukemia cells (13). Recent findings indicate that in U937 human leukemia cells, FP triggers cell death through mitochondrial release of cYc and independently of receptor-mediated procaspase-8 activation (14). Although FP by itself has been shown to induce maturation, e.g., in small cell lung cancer cells (15), it does not necessarily potentiate the actions of established differentiation-inducing agents. For example, when combined with PMA, a PKC activator and tumor promoter that potently induces terminal differentiation in human myeloid leukemia cells (e.g., HL-60 and U937; Refs. 16, 17), FP blocks rather than promotes maturation (18). Instead, it interferes with a variety of differentiation-related events, particularly induction of the CDKI p21cip1, which culminates in caspase activation and cell death. Similar interactions have been observed between FP and the histone deacetylase inhibitor sodium butyrate (19), which also induces leukemic cell maturation (20). Interestingly, whereas PMA potently induces leukemic cell maturation (16, 17), it has also been observed to induce apoptosis in U937 and K562 cells by triggering TNF-a production and release (21).

In this context, we have recently reported that in human leukemia cells, PMA/FP-mediated apoptosis is characterized by early cYc redistribution, followed by a subsequent PKC-dependent increase in TNF-a transcription and release, culminating in procaspase-8 activation and potentiation of cell death (22). Collectively, these findings suggest that FP disrupts PMA-mediated maturation in human leukemia cells, causing cells to engage an alternative TNF-a-related cell death program.

The macrocyclic lactone bryostatin 1, like PMA, also binds to and activates PKC (23). However, its spectrum of activity is distinctly different from that of the phorbol esters. This phenomenon may reflect differential effects of bryostatin 1 on PKC isoform activation (24), nuclear translocation (25), or PKC down-regulation (26). Bryostatin 1, unlike PMA, is a nontumor promoter (27) and is a considerably weaker inducer of leukemic cell differentiation than PMA (28, 29). In fact, in human promyelocytic leukemia cells (HL-60), bryostatin 1 has been shown to block PMA-related differentiation induction (30). The mechanism underlying the relative inability of bryostatin 1 to trigger leukemic cell differentiation is not known but is likely related to its failure to induce the CDKI p21cip1, dephosphorylation of pRb, and arrest of cells in G0-G1 (27).

If as recently postulated (18), the synergistic induction of apoptosis in human leukemia cells by FP and PMA represents a consequence of disruption of the maturation process, then FP should not interact synergistically with bryostatin 1, which is poor differentiation-inducer. Furthermore, because arrest of cells in G0-G1 is essential to the maturation process (31), the possibility arises that a CDKI such as FP might restore the ability of bryostatin 1 to initiate leukemic cell differentiation. To test these possibilities, the effects of FP have been examined with respect to the response of human leukemia cells (U937 and HL-60) to bryostatin 1. Here, we report that coadministration of FP with bryostatin 1, as in the case of PMA, does not promote leukemic cell maturation. Instead, combined treatment with FP and bryostatin 1 is associated with increased mitochondrial damage (e.g., cYc release) resulting from the PKC-dependent release of TNF-a, culminating in enhanced caspase activation and the synergistic induction of apoptosis. Moreover, administration of bryostatin 1 circumvents resistance to FP-mediated apoptosis conferred by ectopic expression of an NH2-terminal phosphorylation loop-deleted Bcl-2 protein, suggesting that perturbations in Bcl-2 phosphorylation status are unlikely to play a role in this phenomenon. Taken together, these findings argue strongly against the notion that FP promotes apoptosis in human myeloid leukemia cells exposed to PKC activators by disrupting the differentiation program but instead indicate that this phenomenon primarily results from enhanced release of TNF-a and subsequent engagement of the extrinsic apoptotic cascade.

Materials and Methods

Drugs and Reagents. Bryostatin 1 was supplied by the National Cancer Institute Cancer Treatment and Evaluation Program courtesy of Dr. Anthony Murgo. Bryostatin 1 was supplied as a sterile powder and was dissolved in DMSO as a 10-4 M stock solution and stored at −20°C. PMA (Sigma Chemical Company, St. Louis, MO) was dissolved in DMSO and 10-5 M stock solution was stored at −20°C. BD-FMK was purchased from Enzyme Systems (Livermore, CA). The mitochondrial dye DiOC6 was purchased from Molecular Probes (Eugene, OR). The human TNF soluble receptor I/Fc chimera (R&D Systems, Inc., Minneapolis, MN) and human TNF-a (Calbiochem, San Diego, CA) were dissolved in sterile PBS containing 0.5% FBS and aliquots stored at −80°C. U0126 (Alexis Biochemicals, San Diego, CA) was dissolved in DMSO and a 10 mM stock solution was stored at −20°C. BisM (Calbiochem) was formulated in DMSO, and a 1-mM stock solution was stored at −20°C. The pancaspase inhibitor BD-FMK was purchased from Enzyme Systems Products and prepared in DMSO according to instructions provided by the manufacturer. Geneticin and Hygromycin were obtained from Life Technologies, Inc. (Grand Island, NY). Primary antibody for actin was purchased from Transduction Laboratories (Lexington, KY). The primary antibodies for PARP, caspase 8, and pRb were purchased from Biomol Research Laboratories, Inc. (Gaithersburg, MD). Coomassie protein assay reagent was purchased from Pierce (Rockford, IL) and an enhanced chemiluminescence kit was obtained from New.
England Nuclear (Boston, MA). Annexin V-FITC was purchased from BD PharMingen. All other chemicals or reagents were from Sigma Chemical Company.

**Cell Culture.** The myelomonocytic leukemia cell line U937 was obtained from American Type Culture Collection. HL-60 cells were derived from a patient with acute promyelocytic leukemia as described previously (32). All cells were cultured in suspension in phenol red-free RPMI 1640 (Life Technologies, Inc.) and 10% (v/v) FCS (HyClone, Logan, UT) and maintained in a humidified atmosphere (95% air/5% CO_2) at 37°C. To obtain FADD-DN (33)-expressing cell lines, U937 cells were transfected by electroporation with pcDNA vector 3.1 (Invitrogen, Carlsbad, CA) containing the appropriate coding region sequences as described previously (34). These cells designated as U937/FADD-DN were maintained along with their empty vector counterparts (U937/pcDNA3.1) as described above in the presence of Genetricin (400 μg/ml). U937 cells ectopically expressing the NH2-terminal phosphorylation loop-deleted (residues 32–80) Bcl-2 protein –10 nM) alone or in combination with FP (100 nM) and bryostatin 1 with FP were characterized as previously described (35). Transfected cell lines were maintained in selection media (Hygromycin or Geneticin; 400 μg/ml) and transferred to selection-free media 24-h before experimentation. All experiments were performed on cells in logarithmic phase.

**Assessment of Drug Interactions.** Interactions between PMA and bryostatin 1 with FP were characterized as previously described by Chou and Talalay (36). The percentage of apoptotic cells was assessed after exposure to PMA or bryostatin 1 (1–10 nM) alone or in combination with FP (100 nM) treatment alone for 24 h using a nonconstant dose ratio design. The CI was determined using Median Dose Effect Analysis Software (Elsevier Biosoft, Cambridge, United Kingdom). CI values < 1 correspond to synergistic drug interactions.

**Western Analysis.** Equal quantities of protein (25 μg/condition) were separated by SDS-PAGE [PARP (8.0%) and procaspase 8 (12%)] and electroblotted onto nitrocellulose. Blots were blocked in TBS-T/5% milk, washed twice with TBS-T, and incubated overnight at 4°C with the appropriate primary antibody. The blots were incubated with a horse-radish-peroxidase-conjugated secondary antibody diluted in TBS-T/5% milk. After incubation, blots were developed by enhanced chemiluminescence exposure to Kodak X-OMAT film and reprobed with antibodies directed against actin to control for equal loading of protein.

**Morphological Assessment of Apoptosis.** Leukemic cells were evaluated for apoptosis by morphological assessment of Wright and Giemsa-stained cytopsin preparations. Cells were transferred to slides by cytocentrifugation, fixed, stained, and evaluated under light microscopy for treatment-induced apoptosis. Apoptotic cells were identified by classical morphological features (i.e., nuclear condensation, cell shrinkage, and formation of apoptotic bodies). Five or more randomly selected fields, encompassing a total of 500 cells/slide, were evaluated to determine the percentage of apoptotic cells for each treatment condition.

**Cyt c Release.** The assay for cyt c release was performed as previously described by Single et al. (37) with modifications. At designated times after drug treatment, 4 × 10⁶ cells were washed with PBS and resuspended in 50 μl of assay buffer (75 mM NaCl, 1 mM NaH₂PO₄, 8 mM Na₂HPO₄, 1 mM EDTA, and 250 mM sucrose) containing digitonin (700 μg/ml). Three min after addition of digitonin assay buffer, cells were pelleted by centrifugation. The supernatants were transferred to tubes containing 5 X Laemmli buffer (50 μl) and boiled for 5 min. Proteins were separated by SDS-PAGE as described above.

**Flow Cytometric Analysis of Apoptosis in Annexin V/PI-treated Cells.** After drug treatments, cells (1 × 10⁶) were costained with annexin V conjugated to FITC and PI per instructions provided by the manufacturer (BD PharMingen). Samples were analyzed using a Becton Dickinson FACScan flow cytometer. The percentage of apoptotic cells was determined by flow cytometric analysis. This assay is based on the premise that phosphatidylserine externalization to the outer leaflet of the plasma membrane is an early event in apoptosis (38) and allows for discrimination between apoptotic (annexin V positive, A⁺) and necrotic (PI positive, PI⁺) cells.

**Assessment of Mitochondrial Membrane Potential.** At designated intervals, 1-ml aliquots of cells (2 × 10⁶) were harvested and incubated with 40 nM DiOC6 for 15 min at 25°C as described previously (39). Samples were analyzed using a Becton Dickinson FACScan flow cytometer. Results were expressed as the percentage of total cells exhibiting loss of mitochondrial membrane potential (Δψm), manifested by a reduction in DiOC6 uptake relative to untreated controls. Data acquisition and analysis was performed using Cellquest Software (Becton Dickinson, Mansfield, MA).

**ELISA.** U937 cells (2 × 10⁶) were exposed to drug treatment at early (2–9 h) and late (18 h) time points after which the cells were pelleted. Cell culture supernatants were collected and flash frozen for storage at −80°C. The culture supernatants were tested for the presence of TNF-α by the ELISA OptEIA kit (BD PharMingen) according to the protocol provided. Data were normalized to live cell number (2 × 10⁶) to reflect the toxicity of various treatments relative to untreated controls.

**Cell Adherence.** Differentiation was monitored by determining the percentage of U937 cells exhibiting plastic adherence after drug treatment. The percentage of adherent cells was determined as previously described in detail (28).

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

**Results**

**Cotreatment with Bryostatin 1 and FP Results in the Synergistic Induction of Apoptosis in U937 Human Leukemia Cells.** To characterize interactions between bryostatin 1 and FP, induction of apoptosis was assessed in U937 cells exposed for 24 h to a range of bryostatin 1 concentrations (i.e., 0.5–50 nM) in the presence or absence 100 nm FP (Fig. 1). In each case, coadministration of FP with bryostatin 1, exposures that were minimally toxic by themselves, resulted in a marked increase in cell death. CI values for these combinations were <1 for each dose of bryostatin 1.
Characterization of the interactions between FP and bryostatin 1 or PMA in U937 cells

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<tr>
<th>Dose (nM)</th>
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<td>0.5/100</td>
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A nonconstant dose ratio design was used to assess interactions between varying concentrations of bryostatin (B) 1 or PMA (P) (0.5–50 nM each) and a fixed concentration of FP (100 nM) after a simultaneous 24-h drug exposure. At the end of the incubation period, the percentage of apoptotic cells was determined by examining Wright Giemsa-stained cytospin preparations. CI values were determined by median dose effect analysis as described in “Materials and Methods.” CI values < 1.0 correspond to synergistic drug interactions. Results are representative of more than or equal to four separate experiments.

A, PMA and bryostatin 1 exert differential effects on the maturation response of U937 human leukemia cells. Maturation was assessed by monitoring the percentage of adherent cells and cells remaining in suspension after a 72-h exposure to 10 nM bryostatin 1 ± 10 nM PMA as described in “Materials and Methods.” Values represent the means for three separate experiments ± SE. B, FP blocks PMA-related maturation and fails to promote bryostatin 1-induced differentiation in the presence of caspase inhibition. U937 cells were exposed to 10 nM bryostatin 1 or PMA for 12 h ± FP (100 nM) in the presence or absence of the pan-caspase inhibitor BD-FMK (inh; 25 μM). At the end of the incubation period, the percentage of adherent cells was determined as above. Values represent the means for more than or equal to four separate experiments ± SE.

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Fig. 1. Characterization of the interactions between bryostatin 1 (B) and FP. The percentage of apoptotic cells was monitored after a 24-h exposure of U937 cells to a range of (B) concentrations (0.5–50 nM) alone and in combination with 100 nM FP (B/FP). Apoptosis was determined by morphological assessment of cytospin preparations as described in “Materials and Methods.” Values represent the means for five or more separate experiments ± SE.
In view of evidence that bryostatin 1 in-
feres with procaspase-8 activation at the level of the DISC. Consistent with earlier
results (22), U937/FADD-DN cells exhibited marked resistance to TNF/CHX-induced apoptosis but remained fully sensi-
tive to cell death induced by VP-16, which is primarily
mediated through the intrinsic mitochondrial-dependent
pathway (Fig. 4). Notably, bryostatin 1/FP-induced lethality was significantly attenuated in U937/FADD-DN cells com-
pared with their empty vector counterparts based on both
morphological features (i.e., 25 ± 1% versus 60 ± 2%; P <
0.001) as well as annexin V/PI positivity (P < 0.005 versus
control). Taken together, these data suggest that recep-
tor-mediated events, particularly FADD activation, are critical for bryostatin 1/FP-induced apoptosis in leukemic cells.

Enhanced Apoptosis in Bryostatin 1/FP-treated Cells Is
Associated with a Delayed PKC-dependent Increase
in TNF-α Release. In view of evidence that bryostatin 1 in-
duces TNF-α release (41, 42), TNF-α protein levels were
monitored by ELISA after treatment of U937 cells with bryo-
statin 1 or FP (Fig. 5A). Although FP by itself did not induce
TNF-α, bryostatin 1 triggered an initial increase in TNF-α
levels at 6 h (e.g., 7-fold greater than control values), which
returned toward baseline values over the ensuing 6 h (e.g., to
only 2-fold greater than controls at 12 h). In contrast, the
combination of bryostatin 1/FP triggered a more modest
increase in TNF-α levels at 6 h (e.g., 3-fold greater than
controls), but this increase was sustained (e.g., 5-fold greater
than basal levels after 12 h). At the latter interval, TNF-α
levels were significantly higher than those observed with
bryostatin 1 alone (P < 0.04). Furthermore, the specific PKC
inhibitor BisM (2 μM) completely abrogated TNF-α release
triggered by the combination of FP and bryostatin 1, con-
sistent with the results of previous studies linking TNF-α
release to PKC activation (43, 44).

To assess the functional significance of these events,
U937 cells were exposed to bryostatin 1/FP in the presence
or absence of TNFSRs, which block TNF-mediated lethality
(45). As anticipated, addition of TNFSRs essentially abro-
gated apoptosis induced by TNF-α + CHX (Fig. 5B). More-
over, TNFSRs significantly attenuated bryostatin 1/FP medi-
ated cell death i.e., from 62 ± 2% to 24 ± 4% at 24 h (P <
0.02). Western blot analysis confirmed that TNFSRs largely
blocked procaspase-8 and PARP cleavage in TNF/CHX-
treated cells as well as in cells exposed to bryostatin 1/FP,
although to a slightly lesser extent (Fig. 5C). Collectively,
these findings argue strongly that synergistic induction of
apoptosis by bryostatin 1 and FP proceeds, at least in part,
through TNF receptor-mediated events.

Interruption of PKC- and MEK1/2-related Signaling
Events Markedly Attenuate Bryostatin 1/FP-mediated
Apoptosis in U937 Cells. In view of evidence that the en-
hanced release of TNF-α in bryostatin 1/FP-treated cells is
PKC dependent (Fig. 6A), the functional significance of this
event with respect to lethality was assessed using the se-
lective PKC antagonist BisM. Parallel studies were per-
formed using the MEK1/2 inhibitor U0126 (46). Administra-
tion of BisM (2 μM) or U0126 (20 μM) with bryostatin 1/FP
resulted in a substantial reduction in cell death, reflected by
morphological criteria (Fig. 6A) or diminished annexin V/PI
positivity (data not shown; P < 0.001 in each case). Similar
reductions were observed when loss of mitochondrial mem-
brane potential (ΔΨm) and PARP degradation were moni-
tored (Fig. 6, B and C). Collectively, these and the preceding find-
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Alterations in TNF-α/FP primarily activates the intrinsic apoptotic pathway in U937 cells. The symbol * denotes values significantly less than those obtained after combined treatment with bryostatin and FP; P < 0.001 in each case. B, loss of mitochondrial membrane potential (Δψm) was monitored in U937 cells after treatment as described above. Values represent the percentage of cells exhibiting low uptake of the fluorochrome DiOC6. The symbols + and ++ denote values significantly less than those obtained in cells exposed to the combination of bryostatin 1 and FP; + = P < 0.04; ++ = P < 0.01, respectively. For A and B, values represent the means for three separate experiments ± SE. C, Western blot analysis of PARP degradation after 24-h exposure of U937 cells to the agents as described above. CF corresponds to a M, 85,000 PARP cleavage fragment. Blots were reprobed with actin to ensure equivalent protein loading and transfer. An additional experiment yielded similar results.

Interruption of the TNF and PKC Pathway Blocks Bryostatin 1/FP-mediated Cyt c Release in U937 Cells. To gain insights into the hierarchy of events that accompany bryostatin 1/FP-mediated apoptosis, effects of TNFSRs and BisM were examined in relation to release of cyt c, an early event in the apoptotic process (Fig. 7). Bryostatin 1 alone triggered a modest release of cyt c into the cytosol at 6 h, whereas FP was more effective in this regard. The latter finding is consistent with previous results demonstrating that FP primarily activates the intrinsic apoptotic pathway in U937 cells (14). However, combined exposure of U937 cells to
bryostatin 1 and FP resulted in a marked increase in release of cyt c. This effect is qualitatively similar to that previously observed in cells exposed to FP and PMA (18, 22). However, coexposure of cells to bryostatin 1/FP and either TNFSRs or BisM largely abrogated cyt c release into the cytosol. These findings imply that the PKC-dependent induction of TNF-α is largely responsible for or contributes to cyt c release, and by extension, activation of the intrinsic apoptotic cascade.

TNF Receptor-related Pathways Contribute to the Synergistic Induction of Apoptosis by Bryostatin 1 and FP in Promyelocytic Leukemia Cells (HL-60). To determine whether PKC-dependent TNF receptor-related pathways played a role in bryostatin 1/FP-induced apoptosis in leukemia cells other than U937, parallel studies were conducted in human promyelocytic HL-60 cells (Fig. 8, A and B). As shown in Fig. 8A, TNFSRs significantly attenuated bryostatin 1/FP-induced apoptosis in HL-60 cells (i.e., from 59 ± 5% to 33 ± 4%; P < 0.009), whereas BisM (2 μM) reduced lethality to 16 ± 5% (P < 0.001). Equivalent results were obtained when annexin V/PI positivity was used to monitor apoptotic cell death (data not shown). Consistent with these results, TNFSRs or BisM diminished degradation of full-length PARP in HL-60 cells exposed to bryostatin 1/FP (Fig. 8B). These findings indicate that PKC-dependent TNF receptor-mediated events contribute significantly to the synergistic induction of apoptosis by bryostatin 1 and FP in leukemia cells other than U937.

A NH2-Terminal Phosphorylation Loop-deleted Bcl-2 Protein Fails to Protect U937 Cells from Apoptosis Induced by Bryostatin 1/FP. In previous studies, FP (200 nM) effectively induced apoptosis in U937 cells that overexpress full-length Bcl-2, whereas U937 cells ectopically expressing a mutant protein lacking the NH2-terminal Bcl-2 phosphorylation loop domain, i.e., U937.Bcl-2Δ, were highly resistant to this agent (47). Cells expressing this mutant protein are also resistant to apoptosis induced by growth factor deprivation and by a variety of other cytotoxic drugs (35, 48, 49). Therefore, attempts were made to determine whether ectopic expression of U937.Bcl-2Δ would modify the response of U937 cells to bryostatin 1/FP. As shown in Fig. 9, 100 nM FP, 10 nM bryostatin 1, or 10 nM PMA alone induced a very modest degree of apoptosis in parental or U937.Bcl-2Δ cells, although attenuation of PARP cleavage was noted in the mutant line (Fig. 9B). Interestingly, although U937.Bcl-2Δ cells were highly resistant to VP-16-induced apoptosis (P < 0.007), these cells were sensitive to cell death induced by FP + either bryostatin 1 or PMA, as well as by TNF/CHX. Parallel results were obtained when PARP degradation was monitored (Fig. 9B). In separate studies, ectopic expression of full-length Bcl-2 was ineffective in protecting cells from FP/ bryostatin 1-mediated apoptosis (data not shown) or to FP/ PMA-mediated lethality, as described previously (18). Taken together, these observations suggest that the capacity of the bryostatin 1/FP regimen to overcome resistance conferred by ectopic expression of a Bcl-2 phosphorylation loop-deleted protein stems from activation of the TNF-α pathway.

Discussion

The results of this study indicate that synergistic induction of apoptosis in human myeloid leukemia cells by bryostatin 1 and FP largely stems from the TNF-α-dependent activation of the extrinsic apoptotic pathway. Although in some cell types, FP-mediated apoptosis has been attributed to activation of the extrinsic pathway (14), in human leukemia cells such as U937, it appears to act primarily through release of cyt c from the mitochondria and independently of receptor-mediated prosapase-8 activation (14). On the other hand,
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Fig. 9. Coadministration of FP with bryostatin 1 or PMA markedly potentiates apoptosis in U937 cells ectopically expressing a Bcl-2 NH₂-terminal loop-deleted protein (i.e., U937/Bcl-2Δ). A, empty vector U937/psFFV and U937/Bcl-2Δ cells were exposed to 10 nM bryostatin 1 (B) or PMA (P) in the presence or absence of 100 nM FP for 24 h. For controls, cells were exposed to TNF (10 ng/ml) + CHX [1 μM, TNF/cycloheximide (T/C)] or VP16 (50 μM) for 4 h. The percentage of apoptotic cells was quantified by morphological analysis of Wright-Giemsa-stained cytospin preparations as described in "Materials and Methods." Values represent the means for three or more separate experiments ± SE. *P < 0.001. B, Western blot analysis of PARP degradation in U937/psFFV and U937/Bcl-2Δ cells after 24-h exposure to drugs as described above. CF corresponds to a M, 85,000 PARP cleavage fragment. Each lane contained 20 μg of protein; blots were reprobed for actin to ensure equivalent loading and transfer of protein. An additional experiment yielded identical results.

both PMA and bryostatin 1 have been shown to induce TNF-α production and release (21, 41, 42), and in the case of PMA, these events have been specifically linked to apoptosis induction (21). Recently, we have reported that FP markedly enhances PMA-mediated apoptosis through activation of both the extrinsic, receptor-related as well as the intrinsic, mitochondrial-dependent apoptotic pathways (22). The findings that expression of dominant-negative FADD-DN, as well as coadministration of TNFSRs (45) substantially attenuated the apoptotic response to bryostatin 1/FP argue strongly that activation of the TNF receptor pathway plays a major role in bryostatin 1/FP-induced lethality as well. It is important to note that induction of mitochondrial damage, including cyt c release, by certain cytotoxic drugs can be enhanced by proapoptotic activation and Bid cleavage, thereby amplifying the cell death process (3). Indeed, coadministration of bryostatin 1 and FP resulted in the early release of cyt c into the cytosol. However, this release was substantially blocked by TNFRs and PKC inhibitors, indicating that in the case of bryostatin 1/FP-induced apoptosis, engagement of the extrinsic apoptotic cascade represents a primary rather than a secondary phenomenon, and operates upstream of cyt c redistribution.

The observation that bryostatin 1 and PMA interact in a similar manner with FP in human leukemia cells provides insights into the mechanism underlying synergism between these agents. For example, in leukemic cells, differentiation and apoptosis often represent alternative cellular fates (50), and dysregulation of the maturation process, particularly induction of the CDKI p21\(^{CIP1}\), is known to convert a maturation stimulus into an apoptotic one (51, 52). Moreover, FP has been shown to bind to duplex DNA (53) and to down-regulate expression of the antiapoptotic protein Mcl-1 (54). The latter action may reflect the ability of FP to block transcription through inhibition of CDK-9 and the cyclin T complex (pTEFb; Ref. 11). In view of these observations, it was tempting to speculate that FP, by disrupting the normal differentiation process, most notably induction of p21\(^{CIP1}\), converted the response of leukemic cells from maturation to cell death. However, for several reasons, this model is highly unlikely to be operative in the case of bryostatin 1. First, bryostatin 1 is a relatively weak inducer of leukemic cell maturation (28) and, as noted previously, opposes the maturation-inducing actions of PMA (30). Moreover, FP interacted synergistically with the combination of bryostatin 1 and PMA, although coadministration of these agents abrogated differentiation (Fig. 3A). In addition, bryostatin 1 is impaired in its ability to trigger p21\(^{CIP1}\) induction, pRb dephosphorylation, and G₀-G₁ arrest in leukemic cells (28). Thus, the possibility that FP potentiates bryostatin 1-mediated apoptosis in leukemic cells by disrupting the differentiation program appears remote. On the other hand, the ability of FP to promote sustained TNF-α release and activation of the extrinsic apoptotic pathway in cells exposed to either bryostatin 1 (this study) or PMA (22) represents a common feature that could plausibly account for synergistic antileukemic interactions between these agents. In this context, synergistic induction of apoptosis in leukemia cells by FP and the differentiation-inducers/histone deacetylase inhibitors sodium butyrate (19) and suberoylanilide hydroxamic acid (55) have recently been observed. However, in marked contrast to results involving PKC activators, e.g., bryostatin 1 (this study) and PMA (22), FP/suberoylanilide hydroxamic acid and FP/sodium butyrate-mediated lethality was not attenuated by TNFSRs (55). Such findings suggest that activation of the extrinsic apoptotic pathway in cells exposed to FP in conjunction with maturation-inducing agents varies with the differentiation inducer.

The capacity of FP to down-regulate expression of the antiapoptotic protein Mcl-1 could also contribute to the enhanced lethality of the bryostatin 1/FP regimen. Mcl-1 has been shown to play an important role in the survival of malignant hematopoietic cells, including myeloma (56) as well as myeloid leukemia cells (40). In this regard, FP-induced apoptosis in myeloma (54) and chronic lymphocytic leukemia cells (57) has been related to Mcl-1 down-regulation. In accord with these findings, reduction of Mcl-1 expression (e.g., by an antisense strategy) has been shown to promote apoptosis in U937 cells undergoing maturation in response to PMA (40). Although bryostatin 1 is a poor inducer of differentiation in U937 cells and hence a relatively weak inducer of Mcl-1 expression (i.e., compared with PMA),

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4 R. Rosato and S. Grant, unpublished results.
it mimics the ability of PMA to trigger TNF-α release. Consequently, FP-mediated down-regulation of Mcl-1 expression could provide a common mechanism by which this agent lowers the threshold for bryostatin 1 and PMA-induced mitochondrial injury and apoptosis.

The observation that the PKC inhibitor BisM to blocked TNF-α secretion in bryostatin 1/FP-treated cells indicates that in this setting, TNF-α modulatory events depend upon PKC activation. This finding is consistent with previous reports suggesting that PKC activation is critical for the induction of multiple inflammatory cytokines such as TNF-α and interleukin 1β (43, 58, 59). In addition, activation of TNF-α-converting enzyme, which is responsible for cleaving pro-

References


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Protein Kinase C-dependent Activation of the Tumor Necrosis Factor Receptor-mediated Extrinsic Cell Death Pathway Underlies Enhanced Apoptosis in Human Myeloid Leukemia Cells Exposed to Bryostatin 1 and Flavopiridol

Leanne Cartee, Sonia C. Maggio, Rebecca Smith, et al.


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