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Synergistic Induction of Apoptosis in Human Myeloid Leukemia Cells by Phorbol 12-Myristate 13-Acetate and Flavopiridol Proceeds via Activation of Both the Intrinsic and Tumor Necrosis Factor-Mediated Extrinsic Cell Death Pathways

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ABSTRACT

Previous studies have shown that coexposure to marginally toxic concentrations of phorbol 12-myristate 13-acetate (PMA; 10 nM) and the cyclin-dependent kinase inhibitor flavopiridol (FP; 100–200 nM) synergistically induces apoptosis in human myeloid leukemia cells U937 and HL-60 (i.e., >50% apoptotic at 24 h). Attempts have now been made to characterize the cell death pathway(s) involved in this phenomenon. In contrast to cytochrome *c* release and caspase-3 activation, which occur within 2.5 h of PMA/FP coexposure, caspase-8 activation and Bid cleavage appeared as later events. Such findings implicate the mitochondria-dependent pathway in the initial induction of apoptosis by PMA/FP. However, U937 cells ectopically expressing CrmA, dominant-negative caspase-8, or dominant-negative Fas-associated death domain that were highly resistant to tumor necrosis factor (TNF)/cycloheximide-induced lethality displayed significant, albeit incomplete, resistance to PMA/FP-induced apoptosis after 24 h. Furthermore, coadministration of TNF soluble receptor significantly attenuated PMA/FP-induced apoptosis in U937 ($p < 0.02$) and HL-60 ($p < 0.03$) cells at 24 h. PMA/FP coadministration also triggered substantial increases in TNFα mRNA and protein secretion compared with the effects of PMA administered alone. The protein kinase C (PKC) inhibitor bisindolylmaleimide (1 μM) completely blocked PMA/FP-induced TNFα secretion in U937 cells and attenuated apoptosis. Taken together, these results suggest that coadministration of PMA with FP in myeloid leukemia cells initially triggers mitochondrial damage, an event followed by the PKC-dependent induction and release of TNFα, supporting a model in which the synergistic induction of leukemic cell apoptosis by this drug combination proceeds via both mitochondrial- and TNF receptor-related apoptotic pathways.

Apoptosis was originally described as a series of morphological changes exhibited by dying cells in biological systems (Kerr et al., 1972). It is a highly conserved process of cell suicide that involves the activation of a family of cysteine proteases known as caspases (Earnshaw et al., 1999). Caspases are the primary executioners of apoptosis, and their activation is responsible for the characteristic biochemical and morphological features displayed by dying cells in response to a variety of stimuli (Hengartner, 2000). Apoptosis proceeds via two distinct biochemical caspase cascades designated as intrinsic and extrinsic pathways (Budihardjo et al., 1999; Sun et al., 1999). The intrinsic or mitochondrial pathway is triggered by ionizing radiation or cytotoxic drugs and is initiated by cytochrome *c* (cyt *c*) release from the mitochondria (Barinaga, 1998). 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, -6, and -7, that promote cell death by initiating DNA fragmentation and intracellular protein degradation (Hengartner, 2000). In con-

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ABBREVIATIONS: cyt *c*, cytochrome *c*; DISC, death-inducing signaling complex; DN, dominant-negative; DN8, procaspase-8-dominant-negative; FADD, Fas-associated death domain; FP, flavopiridol; CDK, cyclin-dependent kinase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; TNF, tumor necrosis factor; DMSO, dimethyl sulfoxide; $DiOC_6$, 3,3-dihexlyoxacarbocyanine; CHX, cycloheximide; PARP, poly(ADP-ribose)polymerase; TUNEL, terminal deoxytransferase-mediated dUTP nick-end labeling; PI, propidium iodide; ELISA, enzyme-linked immunosorbent assay; VP-16, etoposide; BisM, bisindolylmaleimide; TNFSR, tumor necrosis factor soluble receptor; TBST, Tris-buffered saline/Tween 20; RT-PCR, reverse transcriptase-polymerase chain reaction; $\Delta\Psi_m$, loss of mitochondrial membrane potential.

trast, the extrinsic apoptotic pathway is receptor-mediated and involves the recruitment of procaspase-8 to the death-inducing signaling complex (DISC) of cell surface death receptors (Ashkenazi and Dixit, 1998; Wallach et al., 1999). The DISC contains the Fas-associated death domain (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 II receptor-mediated pathways (Scaffidi et al., 1998, 1999). In type I receptor-mediated apoptosis, caspase-8 directly cleaves and activates procaspase-3 (Sun et al., 1999), and cell death proceeds rapidly without a mitochondrial component. In type II receptor-mediated apoptosis (Budihardjo et al., 1999), activated caspase-8 cleaves Bid, yielding a cleavage product tBid (Cheng et al., 2001), which triggers cell death via cyt *c* release and apoptosome formation.

Flavopiridol (FP) is a cyclin-dependent kinase (CDK) inhibitor 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 (Senderowicz, 1999). FP blocks the expression of various cyclins (Carlson et al., 1999) and induces either G_1 and/or G_2-M cell cycle arrest. FP is also an effective inducer of apoptosis in human leukemia cells (Parker et al., 1998) and, based on evidence that disruption of cell cycle progression is a potent apoptotic stimulus (Meikrantz and Schlegel, 1995), its cytotoxicity may stem from cell cycle perturbations (Lundberg and Weinberg, 1999). Aside from studies involving cytotoxic agents (Bible and Kaufmann, 1997), little information is available concerning interactions between FP and other classes of drugs, such as differentiation-inducing agents. Phorbol 12-myristate 13-acetate (PMA) is a protein kinase C (PKC) activator and tumor promoter that induces terminal differentiation in human myeloid leukemia cells (Jiang et al., 1994; Carey et al., 1996). Leukemic cell maturation triggered by PMA requires exit from the cell cycle and G_1 arrest (Jiang et al., 1994). Because FP blocks cell-cycle progression (Lee et al., 1999), we postulated that FP coadministration would potentiate PMA-induced leukemic cell maturation. Contrary to expectations, FP coadministration resulted in dysregulation of various cell-cycle regulatory signaling pathways associated with PMA-induced G_1 arrest and differentiation (Cartee et al., 2001). Furthermore, disruption of PMA-mediated maturation in human leukemia cells (HL-60 and U937) by FP was accompanied by a pronounced increase in apoptosis.

The apoptotic pathways responsible for leukemic cell death induced by PMA/FP cotreatment are currently undefined. Although FP-mediated lethality has been reported to be caspase-8–dependent in cervical carcinoma cells (Achenbach et al., 2000), recent findings indicate that in human leukemia cells (e.g., U937), FP triggers cell death through mitochondrial release of cyt *c* and independently of receptor-mediated procaspase-8 activation (Decker et al., 2001). Interestingly, although PMA induces leukemic cell maturation (Jiang et al., 1994; Carey et al., 1996), it has also been reported to induce apoptosis in U937 and KY321 cells by triggering tumor necrosis factor (TNF)α production and release (Takada et al., 1999). TNFα is a pleiotropic cytokine (Carswell et al., 1975; Wang et al., 1985) that exerts its cytotoxic actions by binding to TNF receptor I (Tartaglia and Goeddel, 1992), a death-inducing receptor present on the surface of all nucleated cell types (Hohmann et al., 1989). Given these findings, the possibility that PMA/FP-mediated lethality might involve the mitochondria-dependent pathway, the TNF receptor-mediated pathway, or both of these cascades, seemed plausible. We reported previously that coadministration of PMA and FP leads to early potentiation of cyt *c* release (Cartee et al., 2001), an event that occurs upstream of caspase activation and precedes procaspase-8 and Bid cleavage. We report here that PMA/FP coadministration also results in a marked PKC-dependent increase in TNFα transcription and release, culminating in TNF receptor-mediated procaspase-8 activation and potentiation of cell death. Taken together, these findings demonstrate that the synergistic apoptosis induced in leukemic cells by PMA/FP coadministration involves activation of extrinsic, TNF-related as well as intrinsic, mitochondria-dependent cell death pathways.

Materials and Methods

Drugs and Biological and Chemical Reagents. PMA (Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO), and aliquots stored at −20°C. FP was kindly provided by Dr. Edward Sausville (Cancer Treatment and Evaluation Program; NCI, Bethesda, MD). FP was formulated in DMSO, and 10^{-2} M stock solutions stored at −20°C. The mitochondrial dye 3,3-dihexlyoxacarbocyanine ($DiOC_6$) was purchased from Molecular Probes (Eugene, OR). The human TNF soluble receptor I/Fc chimera (R & D Systems Inc., Minneapolis, MN) was dissolved in sterile phosphate-buffered saline containing 0.5% fetal bovine serum, and aliquots (100 μg/ml) stored at −20°C. Human TNF-α (Calbiochem; San Diego, CA) was dissolved in sterile phosphate-buffered saline/0.5% fetal bovine serum, and aliquots (10 μg/ml) stored at −80°C. Cycloheximide (CHX) was dissolved in DMSO, and a 1 mM stock solution was stored at −4°C. Bisindolylmaleimide (BisM; Calbiochem) was formulated in DMSO, and a 1 mM stock solution stored at −20°C. Geneticin was obtained from Invitrogen (Carlsbad, CA). Primary antibody for actin was purchased from Transduction Laboratories (Lexington, KY). The primary antibodies for poly(ADP-ribose)polymerase (PARP), Bid, and procaspase-8 were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA), R & D Systems Inc., and BD Biosciences PharMingen (San Diego, CA), respectively. Secondary antibodies conjugated to horseradish peroxidase were obtained from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD). Coomassie protein assay reagent was purchased from Pierce Chemical (Rockford, IL), and an enhanced chemiluminescence kit was obtained from PerkinElmer Life Sciences (Boston, MA). Hypo-osmolar buffer for electroporation was purchased from Eppendorf Scientific, Inc. (Westbury, NY). The RNeasy Mini Kit was obtained from QIAGEN (Valencia, CA), and the DNA-free kit was purchased from Ambion (Austin, TX). Annexin V-fluorescein isothiocyanate was purchased from BD Biosciences PharMingen. All other chemicals or reagents were from Sigma-Aldrich.

Cell Culture. The myelomonocytic leukemia cell line U937 was obtained from American Type Culture Collection (Manassas, VA). HL-60 cells were derived from a patient with acute promyelocytic leukemia as described previously (Grant et al., 1992). All cells were cultured in suspension in phenol red-free RPMI 1640 medium (Invitrogen) and 10% (v/v) fetal calf serum (Hyclone Laboratories, Logan, UT) and maintained in a humidified atmosphere (95% air/5% CO_2) at 37°C. The CrmA, FADD–dominant-negative (DN), and procaspase-8-DN (DN8) inserts were kindly provided by Dr. K. Bhalla (Moffit Cancer Center, University of South Florida, Tampa, FL). To obtain CrmA-, FADD- (Memon et al., 1998), and DN8 (amino acid 377 mutant C → A)-expressing cell lines, U937 cells were transfected by electroporation with pcDNA vector 3.1 (Invitrogen) containing the

appropriate coding region sequences as described previously (Wang et al., 1999). These cells, designated U937/CrmA, U937/FADD-DN, and U937/DN8, were maintained along with their empty-vector counterparts (U937/pcDNA3.1) as described above in the presence of Geneticin (400 μg/ml). Transfectant cell lines were transferred to selection-free media 24 h before experimentation. All experiments were performed on cells in logarithmic phase.

Morphological Assessment of Apoptosis. Leukemic cells were evaluated for apoptosis by morphological assessment of Wright-Giemsa–stained cytospin 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 morphologic 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.

Terminal Deoxytransferase-Mediated dUTP Nick-End Labeling Assay. U937 cells were exposed to drug treatment in the absence or presence of a TNF soluble receptor I/Fc chimera. The cells were transferred to slides by cytocentrifugation and fixed and stained for TUNEL (McGahon et al., 1995) using the In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) according to instructions provided by the manufacturer.

Flow Cytometric Analysis of Annexin V/Propidium Iodide (PI) Positivity. After drug treatments, cells (1×10^6) were costained with Annexin V conjugated to fluorescein isothiocyanate and PI per instructions provided by the manufacturer (BD Biosciences PharMingen). 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 (Savill, 1997) and allows for discrimination between apoptotic (Annexin-V–positive, A^+) and necrotic (PI-positive, PI^+) cells.

Caspase Activity Assay. Caspase activity was measured per instructions provided by the manufacturer using a colorimetric assay kit (Biovision; Palo Alto, CA). Briefly, U937 cells (2×10^6) were exposed to 10 nM PMA/100 nM FP or VP-16 (50 μM) at the designated intervals. Caspase activity in cytosolic extracts was measured by spectrophotometric detection of the chromophore *p*-nitroanilide after its cleavage from the labeled substrate *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide for caspase-3 and Ile-Glu-Thr-Asp-*p*-nitroanilide for caspase-8 (Gurtu et al., 1997).

Western Analysis. Equal quantities of protein (25 μg/condition) were separated by SDS-polyacrylamide gel electrophoresis [PARP (8.0%), Bid (4 to 20% gradient), and procaspase-8 (12%)] and electroblotted onto nitrocellulose. Blots were blocked in TBST/5% milk, washed twice with TBST, and incubated overnight at 4°C with the appropriate primary antibody. The blots were incubated with a horseradish peroxidase-conjugated secondary antibody diluted in TBST/5% milk. After incubation, blots were developed by enhanced chemiluminescence exposure to Kodak X-OMAT film (Eastman Kodak, Rochester, NY) and reprobed with antibodies directed against actin to control for equal loading of protein.

Assessment of Mitochondrial Membrane Potential. At designated intervals, 1-ml aliquots of cells (2×10^5) were harvested and incubated with 40 nM $DiOC_6$ for 15 min at 25°C as described previously (Zamzami et al., 1995). Samples were analyzed using a BD Biosciences (San Jose, CA) FACScan flow cytometer (excitation λ = 488 nm; emission λ = 525 nm). Results were expressed as the percentage of total cells exhibiting loss of mitochondrial membrane potential ($\Delta\Psi_m$) manifested by a reduction in $DiOC_6$ uptake relative to untreated control cells. Data acquisition and analysis were performed using CellQuest Software (BD Biosciences).

Enzyme-Linked Immunosorbent Assay. U937 cells (2×10^6) 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 TNFα by the ELISA OptEIA kit (BD Biosciences PharMingen) according to the protocol provided. Data were normalized to live cell number (2×10^6) to reflect the toxicity of various treatments relative to untreated control cells.

RNA Isolation. U937 cells (5×10^6) were exposed to either no drug, 10 nM PMA, 100 nM FP, or 10 nM PMA and 100 nM FP cotreatment for 0, 3 6, 9, or 12 h, and total RNA was isolated from these respective samples using the Qiagen RNeasy Mini Kit. RNA preparations were treated with DNase using the Ambion DNA-free kit. RNA samples (1 μg) were labeled with ethidium-bromide and subjected to Tris borate-EDTA agarose gel (1%) electrophoresis. Ribosomal bands (28S and 18S) were observed under UV light to verify the quality of total RNA preps.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction. TaqMan One-Step RT-PCR was conducted according to specifications provided by the manufacturer (Applied Biosystems; Foster City, CA). Briefly, oligonucleotide probes were labeled at the 5′ end with 6-carboxyfluorescein and at the 3′ end with the quencher dye *N*,*N*,*N*′-tetramethyl-6 carboxyrhodamine. The following probe and primer sequences for human TNFα were kindly provided by Dr. Gregory Buck (Massey Cancer Center DNA Core Facility, Virginia Commonwealth University, Medical College of Virginia): primers were 5′-CCCCAGGACCTCTCTCAATC-3′ or 5′-CATGGGCTACAGGCTTGTCA-3′, and the probe sequence was 5′-CCCAGGCAGTCAGATCATCTTCTCGAA-3′. Probe and primer sets were designed over intron/exon boundaries to prevent amplification of genomic DNA and tested to ensure that the proper size fragment was generated. A master mix of TaqMan reagents was prepared, and 25 ng of RNA was used per reaction in triplicate preparations. Each tube contained both the *TNFα* gene probe/primer and the human actin control probe/primer. Amplification and detection of specific products was performed with the ABI Prism 7700 sequence detection system (Applied Biosystems) with the following cycle profile: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. The critical threshold cycle (Ct), defined as the cycle at which the fluorescence becomes detectable above background, was established manually to ensure that correlation coefficients of the standard curves were between 0.99 and 1.00. A standard curve was plotted for each primer/probe set with Ct values obtained from amplification of known quantities of RNA, and results for the experimental gene were normalized to actin levels as specified by the manufacturer.

Results

PMA/FP-Induced Apoptosis in U937 Cells Increases Progressively over 24 h and Is Initially Mediated by the Mitochondria-Dependent, Intrinsic Pathway. The time course of apoptosis induction in U937 cells after combined exposure to PMA (10 nM) and FP (100 nM) is shown in Fig. 1A. As demonstrated in Fig. 1A and in previous studies, exposure of cells to these agents individually for 24 h exerted minimal toxicity (e.g., <10% apoptotic cells; Cartee et al., 2001). The extent of cell death induced by PMA/FP cotreatment was initially modest in that only 18% of cells were apoptotic after 6 h. However, cell death increased progressively to 45% after 12 h and ultimately reached a level of 66% by 24 h. The gradual onset of cell death is compatible with the notion that new protein synthesis is required for PMA/FP-induced lethality. Because biochemical indices of apoptosis may precede morphological changes, activation of caspase-3 and -8 was monitored at early intervals (Fig. 1B). These studies revealed a significant increase in caspase-3 but not caspase-8 activity 2.5 h after PMA/FP coadministration. By 4 h, activation of caspase-8 was noted, but the extent was less

than that observed in the case of caspase-3. Furthermore, levels of caspase-3 and -8 activity after 4-h PMA/FP exposure were similar to those induced by treatment with the topoisomerase inhibitor VP-16 (50 μM), an agent known to trigger cell death through the intrinsic pathway, resulting in activation of caspase-3 before that of caspase-8 (Sun et al., 1999; Engels et al., 2000). Consistent with these observations, there was no evidence of Bid cleavage after 3 h of PMA/FP cotreatment, in marked contrast to the complete disappearance of this protein in cells exposed to TNF/CHX (Fig. 1C). These findings, along with our previous observation that cyt *c* release occurs independently of caspase activation and within 2 h of PMA/FP cotreatment (Cartee et al., 2001), suggest that activation of the mitochondria-dependent, intrinsic pathway is primarily responsible for PMA/FP-mediated cell death at early intervals (<6 h). However, by 6 h, the relative increase in caspase-8 activity was comparable with that of caspase-3 (Fig. 1B). Moreover, Bid cleavage was apparent in PMA/FP-treated cells at 12 h and quite pronounced after 24 h (Fig. 1C). These findings, as well as previous evidence that PMA induces apoptosis in U937 cells via TNFα production and release (Takada et al., 1999), raised the possibility that receptor-mediated events might be involved in PMA/FP-induced lethality.

U937 Cells Ectopically Expressing CrmA, Dominant-Negative Procaspase-8, or FADD Are Highly Resistant to TNF/CHX-Induced Apoptosis and Are Partially Resistant to PMA/FP-Induced Apoptosis. To evaluate further the possibility that receptor-related events might be implicated in PMA/FP-induced apoptosis, studies were performed in U937 cells ectopically expressing CrmA, a serpin that potently inhibits caspase-8 (Zhou et al., 1997), FADD-DN, or DN8. As shown in Fig. 2, each of these cell lines was highly resistant to TNF/CHX-mediated apoptosis, reflected by markedly diminished procaspase-8 cleavage relative to empty vector control cells (U937/pcDNA3.1). Furthermore, although cells overexpressing CrmA were highly resistant to TNF/CHX-induced apoptosis at 24 h, the extent of protection against PMA/FP-induced apoptosis was substantially less (Fig. 3). Nevertheless, ectopic expression of CrmA significantly protected cells from PMA/FP-mediated apoptosis ($p < 0.01$ relative to empty-vector control cells). PMA/FP-induced mitochondrial damage [i.e., loss of mitochondrial membrane potential ($\Delta\Psi_m$)] was also significantly attenuated by ectopic expression of CrmA (Fig. 3, inset; $p < 0.02$ versus empty-vector control cells).

Similar results were obtained in U937 cells that overexpressed DN8 or FADD-DN (Fig. 4, A-C). Thus, although U937/DN8 and U937/FADD-DN cells exhibited virtually complete resistance to TNF/CHX-induced apoptosis at 24 h, these cells displayed only partial, albeit significant, resistance to PMA/FP-induced apoptosis at this interval (Fig. 4, A and B; $p < 0.04$ versus control in each case). Furthermore, in contrast to their ability to abrogate TNF/CHX-induced procaspase-8 cleavage (Fig. 2), ectopic expression of DN8 or FADD-DN inhibited PMA/FP-mediated procaspase-8 cleavage only partially (Fig. 4C). Parallel results were obtained when PARP cleavage, which primarily reflects caspase-3 activation, was monitored (Fig. 4C). Together, these results suggest that activation of the receptor-related cascade contributes, at least in part, to PMA/FP-mediated cell death. Interestingly, cells expressing DN8 were partially resistant to cell death induced by 50 μM VP-16, whereas cells ectopically expressing FADD-DN, which interferes with procaspase-8 activation upstream at the level of the DISC, were fully sensitive to VP-16–induced apoptosis. Such findings are consistent with previous reports demonstrating that caspase-8 can function as an executioner caspase in certain forms of drug-induced apoptosis initially triggered by mitochondrial injury (Sun et al., 1999; Engels et al., 2000). They also indicate that upstream events, particularly FADD activation, are involved in PMA/FP- but not VP-16–induced cell death.

PMA/FP-Induced Apoptosis Is Partially Attenuated by TNF- Soluble Receptor (TNFSR). Because TNFα pro-

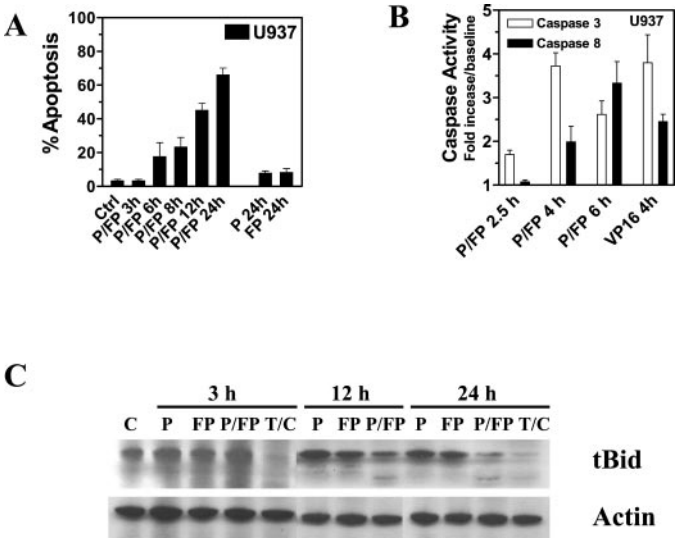

Fig. 1. Effects of PMA/FP coadministration on apoptosis, caspase-3 and -8 activation, and Bid cleavage. A, the percentage of apoptotic cells induced by coadministration of 10 nM PMA (P) and 100 nM FP (FP) over a 24-h interval in U937 monocytic leukemia cells. The extent of apoptosis was determined by morphological analysis of Wright-Giemsa–stained cytospin preparations as described under *Materials and Methods*. Values represent the means for three separate experiments ± S.E. B, the activity of caspase-3 and caspase-8 in U937 cells 2.5 to 6 h after combined treatment with PMA (10 nM) and FP (100 nM). The effects of VP-16 (50 μM) × 4 h are also shown as a control for caspase activation initiated via the intrinsic pathway. Values represent the means for duplicate determinations obtained in three or more separate experiments ± S.E. C, proteolysis of Bid after 3-, 12-, or 24-h exposures to the indicated agents as follows: C, control; *P,* 10 nM PMA; FP, 100 nM FP; P/FP, 10 nM PMA + 100 nM FP; and T/C, TNFα (10 ng/ml) + CHX (1 μM). Bid cleavage was detected by Western analysis as described under *Materials and Methods*. Each lane was loaded with 25 μg of protein; blots were reprobed for actin to ensure equal loading of protein. An additional experiment yielded identical results.

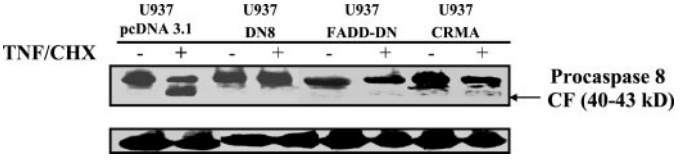

Fig. 2. Ectopic expression of CrmA, dominant-negative FADD, or DN8 blocks TNF/CHX-induced apoptosis. Proteolysis of procaspase-8 into 40- to 43-kDa cleavage fragments (CF) after 6-h treatment with TNFα (10 ng/ml) + CHX (1 μM) in empty-vector control cells (U937/pcDNA3.1) and in cells ectopically expressing CrmA (U937/CrmA), dominant-negative procaspase-8 (U937/DN8), or dominant-negative FADD (U937/FADD-DN). Procaspase-8 cleavage was detected by Western analysis as described under *Materials and Methods*. Each lane was loaded with 25 μg of protein; blots were reprobed for actin to ensure equal loading of protein. An additional experiment yielded equivalent results.

duction and release has been implicated in PMA-related apoptosis (Takada et al., 1999), an attempt was made to determine whether this phenomenon might play a role in PMA/FP-mediated lethality. To this end, U937 cells were exposed for 24 h to PMA (10 nM) and FP (100 nM) in the presence or absence of TNFSR (100 ng/ml), which has previously been shown to oppose TNFα-related lethality (Aggarwal and Natarajan, 1996). As shown in Fig. 5A, PMA/FP-induced mitochondrial damage (i.e., $\Delta\Psi_m$) was significantly attenuated by addition of TNFSRs after 12 h ($p < 0.003$). As anticipated, TNFSRs also substantially blocked TNF/CHX-induced mitochondrial injury. Morphologic assessment of Wright-Giemsa–stained cytospin preparations (Fig. 5B) confirmed that TNFSRs significantly attenuated PMA/FP-induced apoptosis after 24 h [i.e., from 56 to 33% of cells ($p < 0.02$)]. These findings were confirmed by TUNEL analysis, which demonstrated that addition of TNFSRs clearly diminished the percentage of PMA/FP-treated cells displaying DNA strand breaks (Fig. 5D) compared with cells cultured in the absence of TNFSRs (Fig. 5C).

Western blot analysis demonstrated that although addition of TNFSRs completely blocked TNF/CHX-mediated procaspase-8 cleavage at 24 h, partial attenuation of procaspase-8 cleavage was observed in PMA/FP-treated cells (Fig. 6). Parallel results were obtained when PARP degradation was monitored (data not shown). Taken together, these findings argue strongly that the synergistic induction of apoptosis by PMA and FP in U937 cells proceeds, at least in part, through TNF receptor-mediated events.

PMA/FP Coadministration Results in Substantially Greater Levels of TNFα Production and Release Relative to Those Observed in Cells Treated with PMA Alone. In view of evidence linking PMA-induced apoptosis in U937 cells to TNFα production and release (Takada et al., 1999), *TNFα* gene expression was monitored in cells exposed to PMA ± FP using real-time RT-PCR (Fig. 7A). Although FP by itself exerted minimal effects, PMA alone induced a 29-fold increase in TNFα mRNA levels at 3 h, which declined progressively over the ensuing 9 h. Interestingly, although coadministration of FP attenuated PMA-mediated increases in *TNFα* gene expression at 3 h, combined exposure to PMA and FP resulted in a substantial induction of TNFα mRNA at 6 h (~35-fold), which increased to >60-fold over baseline at 9 to 12 h. To determine whether changes in *TNFα* gene expression correlated with effects on protein levels, ELISA assays were performed to monitor TNFα protein released into the medium in response to PMA ± FP (Fig. 7B). Although FP by itself exerted no effect, PMA triggered a transient increase in TNFα protein levels, which were 2-fold greater than control values after 9 h but returned to baseline levels by 18 h. However, the combination of PMA and FP resulted in an even greater release of TNFα protein by 9 h (i.e., >3-fold over baseline); moreover, this increase, in contrast to results obtained with PMA alone, was sustained, resulting in levels ~4-fold over baseline after 18 h ($p < 0.05$ versus PMA alone). In addition, the specific PKC inhibitor BisM (1 μM) completely abrogated TNFα release induced by this drug combination (Fig. 7B). Consistent with this finding, BisM also significantly, albeit partially, reduced PMA/FP-mediated apoptosis [i.e., from 68 to 30% ($p < 0.02$; data not shown)]. Furthermore, immunofluorescence studies using a monoclonal antibody for tumor necrosis factor receptor I in conjunction with flow cytometric analysis indicated that PMA/FP did not significantly alter TNF receptor expression in U937 cells (data not shown). Finally, the protein synthesis inhibitor CHX significantly reduced PMA/FP-induced apoptosis at both 12 h (15 versus 39%, $p < 0.03$) and 24 h (32 versus 66%, $p < 0.003$; Fig. 6C). Collectively, these findings support the

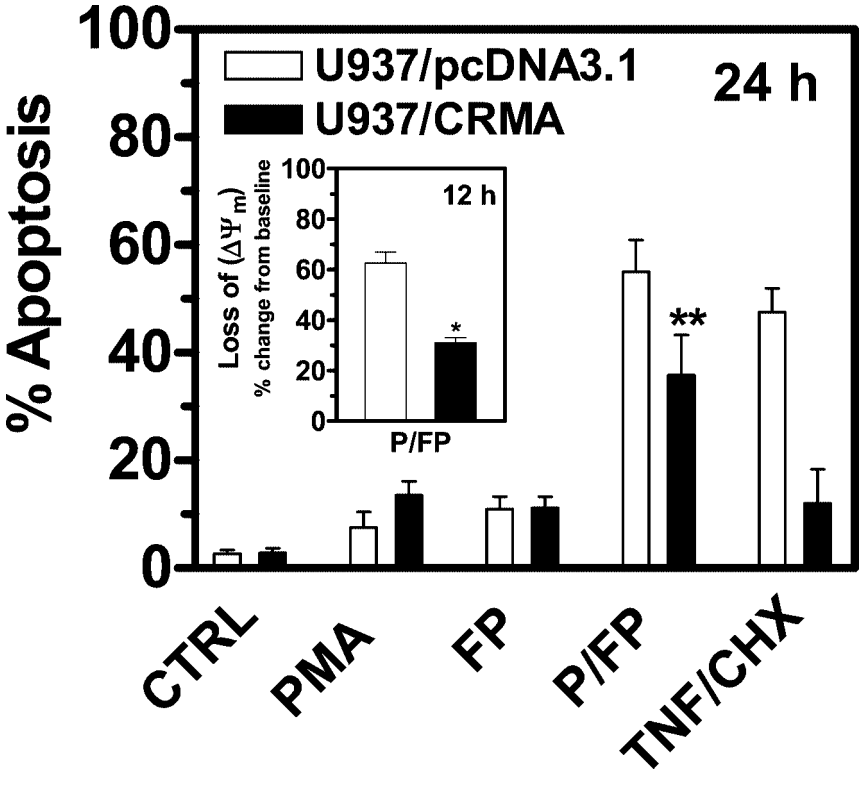

Fig. 3. U937 cells that ectopically express CrmA are highly resistant to TNF/CHX-induced apoptosis and partially resistant to PMA/FP-mediated cell death. U937/pcDNA3.1 and U937/CrmA cells were exposed to PMA (10 nM) + FP (100 nM) or TNFα (1 ng/ml) + CHX (1 μM) for 24 h, after which apoptotic cells were quantified as described under *Materials and Methods*. Values represent the means for four separate experiments ± S.E. In addition, $\Delta\Psi_m$ was monitored in cells after 12-h exposure to PMA and FP as above (Fig. 3, inset). Values represent the means for duplicate determinations from two separate experiments ± S.E. Ectopic expression of CrmA significantly reduced PMA/FP-related mitochondrial damage at 12 h (⋆, $p < 0.01$) and apoptosis at 24 h (⋆⋆, $p < 0.02$) relative to empty-vector control cells.

concept that the synergistic induction of apoptosis by PMA and FP in human leukemia cells proceeds, at least in part, through the PKC-dependent induction of TNFα.

To determine whether TNF receptor-related pathways played a role in PMA/FP-induced apoptosis in leukemic cells other than U937, parallel studies were conducted in human promyelocytic HL-60 cells. TNFSRs significantly attenuated PMA/FP-induced apoptosis in HL-60 cells from 57 to 40% ($p < 0.01$; data not shown). Consistent with these results, TNFSRs partially blocked degradation of full-length PARP in cells exposed to PMA and FP for 24 h (data not shown). These findings indicate that TNF receptor-mediated events contribute, at least in part, to the synergistic induction of apoptosis by PMA and FP in leukemia cells other than U937.

Discussion

The present study was undertaken to determine whether mitochondria-dependent and/or receptor-related pathways were involved in the synergistic induction of apoptosis by PMA and FP in human myeloid leukemic cells. The rationale for this investigation stemmed from the observations that 1) FP treatment alone has been found to promote cell death in U937 cells through the release of cyt *c* from the mitochondria and independently of receptor-mediated procaspase-8 activation (Decker et al., 2001) and 2) PMA has been shown to induce apoptosis in U937 and KY321 cells by triggering TNFα production and release (Takada et al., 1999). In light of these findings, it seemed plausible that mitochondria-dependent (intrinsic) and/or TNF receptor-mediated (extrinsic) pathways might be responsible for the synergistic induction of apoptosis in leukemia cells by this drug combination. The present results indicate that enforced expression of CrmA, DN8, or FADD-DN provided cells with significant, albeit partial, resistance to PMA/FP-related apoptosis. In addition, coadministration of TNFSRs, which are highly effective in blocking TNF-related lethality (Aggarwal and Natarajan, 1996), partially attenuated the apoptotic response to PMA/FP. Taken together, these findings argue that TNF receptor-mediated apoptosis plays a significant role in PMA/FP-induced lethality. However, the observation that increases in caspase-3 activation occur within 2.5 h of PMA/FP cotreatment and precede caspase-8 activation, as well as our previous finding that cyt *c* release occurs independently of caspase activation and also within 2 h of PMA/FP cotreatment (Cartee et al., 2001), suggests that the mitochondria-dependent intrinsic pathway also plays a critical role in PMA/FP-related lethality, particularly at early exposure intervals (<6 h). Thus, the present and earlier studies support a model of

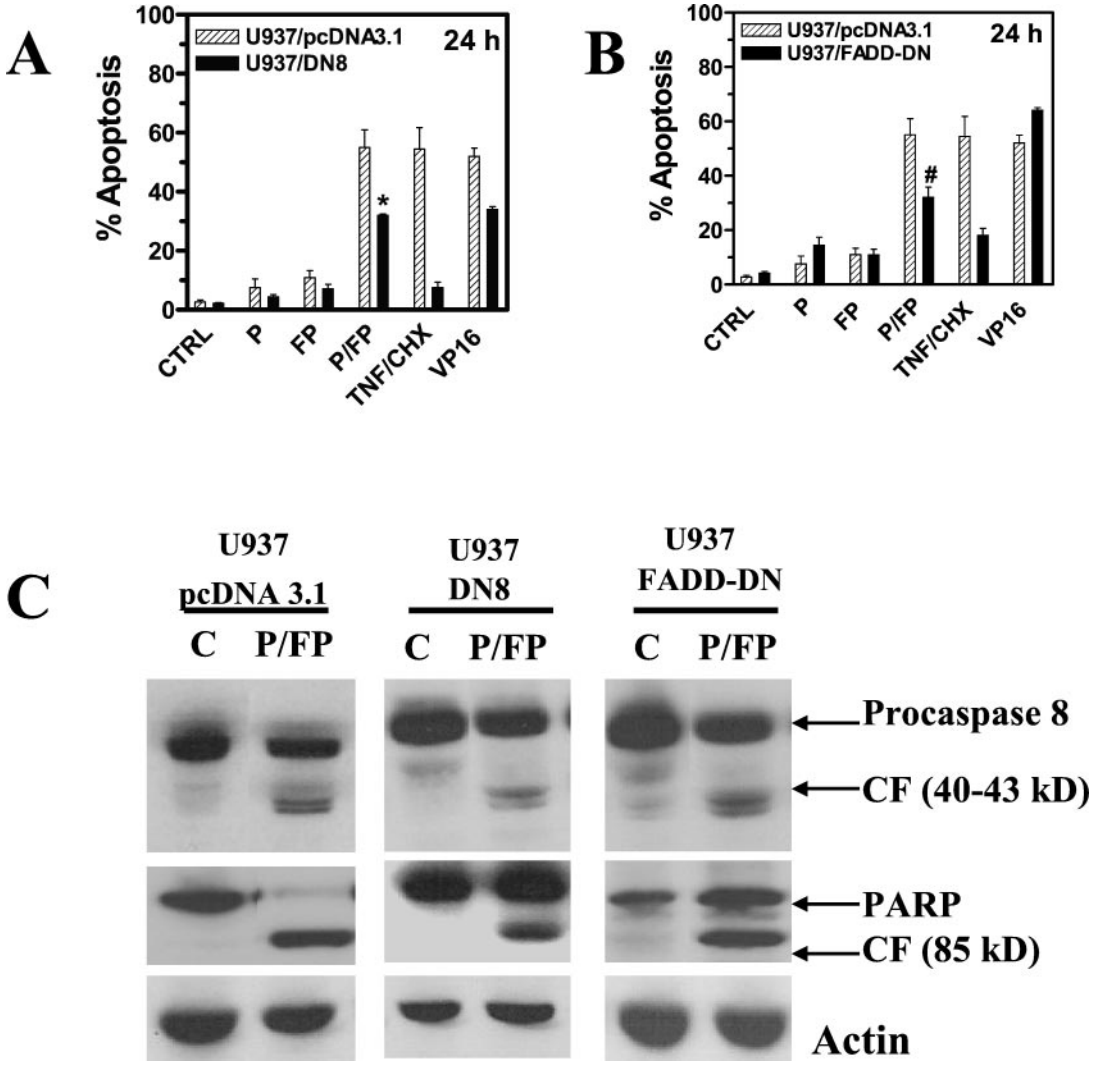

Fig. 4. U937 cells that ectopically express dominant-negative caspase-8 or FADD are highly resistant to TNF/CHX-induced apoptosis and partially resistant to PMA/FP-induced apoptosis. Empty-vector control (U937/pcDNA3.1) and U937/DN8 (A) or U937/FADD-DN (B) cells were exposed for 24 h to drug-free medium (CTRL), 10 nM PMA (P), 100 nM FP (FP), 10 nM PMA +100 nM FP (P/FP), TNF (1 ng/ml) +CHX (1 μM) (TNF/CHX), or VP-16 (50 μM $\times$ 4 h). Apoptotic cells were quantified by morphological analysis of Wright-Giemsa–stained cytospin preparations as described under *Materials and Methods*. Values represent the means for four separate experiments $\pm$ S.E. ($\star$ or #, significantly less than values obtained in empty-vector control cells; $p < 0.04$ in each case). C, cells were exposed to PMA and FP for 24 h as described above, after which degradation of native procaspase-8 (55 kDa) and PARP (116 kDa) to their respective cleavage products (CF) was monitored by Western analysis as described under *Materials and Methods*. Each lane was loaded with 25 μg of protein; blots were reprobed for actin to ensure equal protein loading and transfer. An additional experiment yielded similar results.

PMA/FP-induced apoptosis in which the initial cell death stimulus involves cyt *c* release and caspase-3 activation via the intrinsic pathway, and suggest that these responses are subsequently amplified through a TNF receptor-mediated process.

The finding that FP treatment markedly potentiated PMA-mediated induction of TNFα was unanticipated, and, to the best of our knowledge, represents the first demonstration that a pharmacologic CDK inhibitor promotes elaboration of this cytokine. Aside from evidence implicating TNFα release in PMA-induced apoptosis in U937 cells (Takada et al., 1999), activation of TNF-related pathways has also been linked to induction of retinoic acid-induced apoptosis in promyelocytic leukemia cells (Altucci et al., 2001). Thus, activation of the extrinsic pathway through release of TNFα or related proteins may represent a common mechanism by which apoptosis is induced in leukemic cells undergoing maturation. In this context, we have shown that FP administration results in dysregulation of various signaling and cell cycle regulatory pathways involved in PMA-induced G_1 arrest and leukemic cell differentiation, particularly the ability of FP to block $p21^{WAF1/CIP1}$ induction at the transcriptional level (Cartee et al., 2001). Increased expression of $p21^{WAF1/CIP1}$ contributes to cell cycle arrest in leukemic cells undergoing maturation (Jiang et al., 1994), and dysregulation of this CDK inhibitor is known to disrupt maturation responses (Cartee et al., 2001). Moreover, $p21^{WAF1/CIP1}$ exerts antiapoptotic actions (Asada et al., 1998; Ruan et al., 1998), possibly by binding to and inhibiting procaspase-3, particularly in the case of receptor-related stimuli (Suzuki et al., 1999). Consequently, the balance between maturation and apoptosis in differentiating leukemia cells may be regulated, at least in part, by the

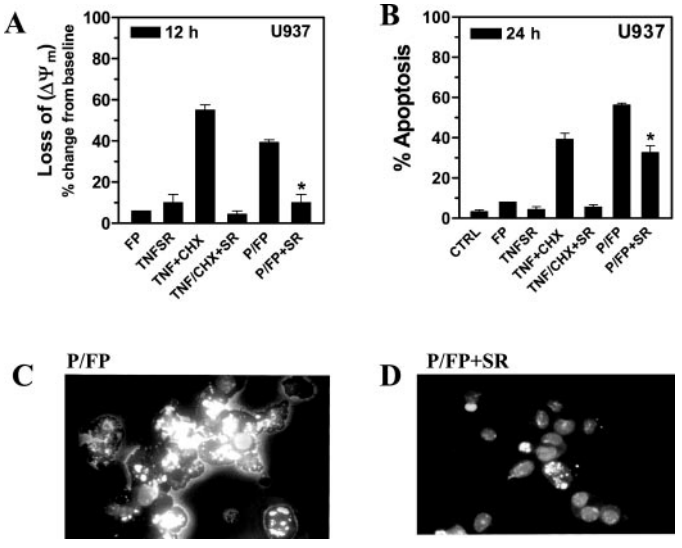

Fig. 5. Coadministration of TNFSR (SR) significantly attenuates PMA/FP-induced mitochondrial damage and apoptosis in U937 cells. A, the loss of mitochondrial membrane potential ($\Delta\Psi_m$) was monitored in U937 cells after 12-h exposure to: FP (100 nM), TNFSR (100 ng/ml), TNFα (1 ng/ml)/CHX (1 μM), TNF/CHX ± TNFSR, and PMA/FP ± TNFSR. Values represent the means for duplicate determinations performed on two separate occasions ± S.E. ⋆, values significantly less than those obtained in cells exposed to PMA/FP in the absence of TNFSRs; $p < 0.003$. B, cells were exposed to the treatments described above for 24 h, after which the extent of apoptosis was determined by morphologic analysis of Wright-Giemsa–stained specimens as described under *Materials and Methods*. Values represent the means for three separate experiments ± S.E. ⋆, significantly less than values obtained for cells exposed to PMA/FP in the absence of TNFSR; $p < 0.02$. TUNEL analysis of cells exposed for 24 h to 10 nM PMA + 100 nM FP (C) or 10 nM PMA/100 nM FP + TNFSR (100 ng/ml) (D), after which photomicrographs (original magnifications, 40×) were taken under fluorescent light. The results of a representative experiment are shown; an additional study yielded equivalent results.

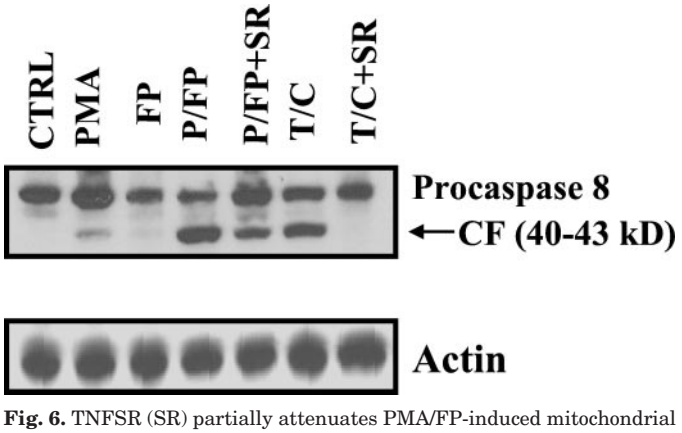

Fig. 6. TNFSR (SR) partially attenuates PMA/FP-induced mitochondrial damage and procaspase-8 cleavage in U937 cells. B, proteolysis of native procaspase-8 to its cleavage fragment (CF) in U937 cells after 24-h exposure to PMA/FP or TNF/CHX in the presence or absence of TNFSR as described above. Procaspase-8 cleavage was monitored by Western analysis as described under *Materials and Methods*. Each lane was loaded with 25 μg of protein; blots were reprobed for actin to ensure equal protein loading and transfer. An additional experiment yielded equivalent results.

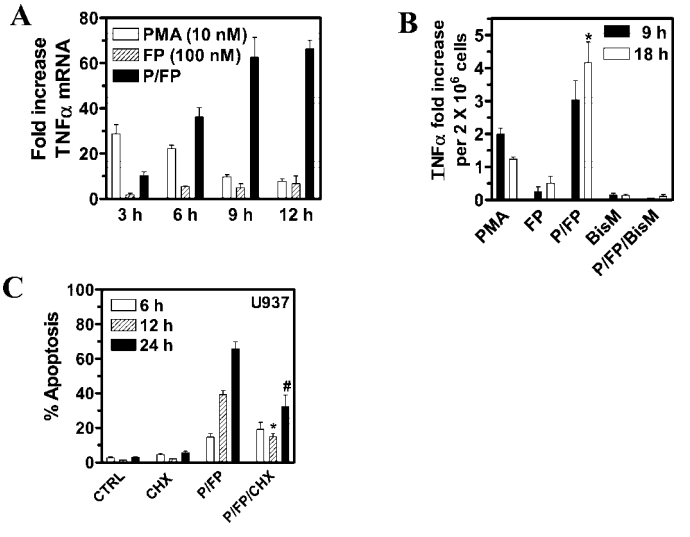

Fig. 7. Treatment with PMA/FP significantly increased TNFα production and release relative to levels observed with PMA treatment alone. A, total RNA was isolated from U937 cells exposed to no drug, 10 nM PMA, 100 nM FP, or 10 nM PMA + 100 nM FP for 3, 6, 9, or 12 h. Real-time RT-PCR was employed to monitor *TNFα* gene expression as described under *Materials and Methods*. The amount of TNFα mRNA was normalized to actin, and the fold increase in mRNA copy number was determined in relation to untreated control cells. B, ELISA was employed to monitor the amount of TNFα protein released into the media after 9 or 18 h of drug treatment as described under *Materials and Methods*. Cells (2×10^6/condition) were exposed to no drug, 10 nM PMA, 100 nM FP, 10 nM PMA/100 nM FP, 1 μM BisM, and 10 nM PMA/100 nM FP + BisM (1 μM). TNFα protein secretion is expressed as fold increase in TNFα, reflected by changes in optical density relative to untreated control cells, per 2×10^6 cells. ⋆, values significantly greater than those obtained for cells treated with PMA alone; $p < 0.05$. C, U937 cells were exposed to PMA/FP ± CHX (1 μM) for 6, 12, or 24 h, after which the percentage of apoptotic cells was determined by morphological analysis of Wright-Giemsa–stained cytospin preparations (⋆, significantly less than values for PMA/FP alone; $p < 0.03$; #, $p < 0.003$). Values represent the means for three separate experiments ± S.E.

respective pro- and antiapoptotic influences of TNFα and p21$^{WAF1/CIP1}$. Thus, the ability of FP to promote PMA-mediated TNFα production and simultaneously block PMA-associated p21$^{WAF1/CIP1}$ induction would be expected to shift the balance away from maturation toward cell death. The mechanism by which FP coadministration ultimately stimulates expression of certain PMA-related genes (e.g., TNFα) and attenuates the expression of others (e.g., p21$^{WAF1/CIP1}$) is unclear. However, such phenomena could be related to the ability of FP to form DNA duplexes (Bible et al., 2000) or inhibit transcription globally (Lam et al., 2001), possibly through blockade of positive transcription elongation factor b binding to CDK 9 (Chao et al., 2000).

There is abundant evidence that cross-talk occurs between the intrinsic and extrinsic cell death pathways and that activation of the latter may amplify responses to stimuli that initially trigger mitochondrial damage. For example, in the case of certain cytotoxic drugs, activation of the mitochondrial pathway can trigger secondary activation of caspase-8, leading, in turn, to Bid cleavage, followed by further mitochondrial damage and cyt *c* release (Sun et al., 1999). Thus, the initial mitochondrial injury induced by PMA/FP could activate the extrinsic pathway as a secondary event, analogous to responses to certain cytotoxic drugs. However, for several reasons, it seems unlikely that such a phenomenon is solely responsible for engagement of the extrinsic pathway in PMA/FP-treated cells. First, coadministration of PMA/FP resulted in a significant and sustained increase in *TNFα* gene expression, accompanied by enhanced TNFα protein secretion. Second, FADD-DN, which acts upstream of caspase-8 activation, significantly attenuated PMA/FP-mediated apoptosis but exerted no effect on VP-16–mediated lethality. Finally, the ability of TNFSR to attenuate, at least in part, PMA/FP-induced lethality as well as caspase-8 activation suggests a specific role for TNFα production and release in the synergistic induction of apoptosis by PMA and FP. Collectively, these findings make it seem unlikely that engagement of the extrinsic apoptotic cascade by PMA/FP simply represents a consequence of initial mitochondrial injury.

The ability of the PKC inhibitor BisM to block TNFα secretion in PMA/FP-treated cells indicates that TNFα modulatory events depend on PKC activation. This finding is consistent with previous reports documenting the role of PKC activation in the induction of multiple inflammatory cytokines, such as TNFα and interleukin-1β (Kontny et al., 2000), as well as evidence relating PMA-induced apoptosis to TNFα production (Takada et al., 1999). Analogously, PMA has been shown to promote cell surface internalization of TNFα-converting enzyme and ectodomain shedding of TNFα (Doedens and Black, 2000). Activation of TNFα-converting enzyme, which is responsible for cleaving pro-TNFα (26 kDa) to the secreted soluble 17-kDa form (Gearing et al., 1994), has been shown to be regulated by PKCδ (Izumi et al., 1998). In view of these considerations, the capacity of a PKC inhibitor, such as BisM, to block PMA/FP-mediated TNFα induction was anticipated. However, in view of recent evidence linking cyt *c* release and apoptosis to mitochondrial translocation of specific PKC isoforms (Li et al., 1999; Majumder et al., 2000), the possibility that BisM may also act to attenuate PMA/FP-mediated mitochondrial injury cannot be excluded.

In summary, the findings presented here suggest that both the TNF receptor-mediated and the intrinsic apoptotic pathways play a role in PMA/FP-induced lethality in leukemic cells. Specifically, caspase-3 activity precedes caspase-8 activation in U937 cells after 2.5 h of PMA/FP coadministration. This observation, along with our previous finding that cyt *c* release occurs independently of caspase activation and within 2 h of PMA/FP cotreatment (Bagchi et al., 1990), suggests that the mitochondria-dependent intrinsic pathway to apoptosis initiates the cell death response at early intervals (<6 h). Furthermore, the present findings indicate that the ability of the CDK inhibitor FP to promote PMA-induced apoptosis in human leukemia cells (U937 and HL-60) involves the PKC-dependent potentiation of TNFα induction/release and activation of the extrinsic, TNF receptor-related pathway at later intervals (>6 h). Recently, considerable attention has focused on strategies designed to activate the extrinsic apoptotic cascade, based on evidence that TNF-related proteins, such as TRAIL (TNF-related apoptosis-inducing ligand), can enhance drug-induced lethality (Cuello et al., 2001) and may also be involved in the lethal actions of certain differentiation-inducing agents (Altucci et al., 2001). One theoretical advantage of this approach is that although Bcl-2 effectively protects cells from noxious stimuli acting through the intrinsic, mitochondrial-related cascade, it blocks activation of the extrinsic cell death pathway relatively ineffectively (Scaffidi et al., 1999). Thus, induction of TNFα may explain, at least in part, the limited ability of ectopic expression of Bcl-2 to protect leukemia cells from PMA/FP-induced lethality (Cartee et al., 2001). Given the recent introduction of PMA and FP into clinical trials in humans (Han et al., 1998; Senderowicz, 1999), further efforts to examine the antileukemic potential of this combination regimen seem warranted.

Acknowledgments

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