Despite the relevant therapeutic progresses made in these last 2 decades, the prognosis of acute myeloid leukemia (AML) remains poor. Phorbol esters are used at very low concentrations as differentiating agents in the therapy of myeloid leukemias. Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), in turn, is a death ligand that spares normal cells and is therefore currently under clinical trials for cancer therapy. Emerging evidence, however, suggests that TRAIL is also involved in nonapoptotic functions, like cell differentiation. PKC\(\varepsilon\) is differentially modulated along normal hematopoiesis, and its levels modulate the response of hematopoietic precursors to TRAIL. Here, we investigated the effects of the combination of phorbol esters (phorbol ester 4\(\beta\)-phorbol-12,13-dibutyrate [PDBu]) and TRAIL in the survival/differentiation of AML cells. We demonstrate here that PDBu sensitizes primary AML cells to both the apoptogenic and the differentiative effects of TRAIL via PKC\(\varepsilon\) down-modulation, without affecting TRAIL receptor surface expression. We believe that the use of TRAIL in combination with phorbol esters (or possibly more specific PKC\(\varepsilon\) down-modulators) might represent a significative improvement of our therapeutic arsenal against AML. (Blood. 2009;113:3080-3087)

Introduction

Although these last 2 decades have seen relevant progresses in the therapy of acute myelogenous leukemia (AML) in terms of cytogenetic prognostic factors, bone marrow transplantation, and targeted therapies,1 the 5-year survival rate is still 20% to 30%2 for adult primary AML, and is even worse for AML arising from myeloproliferative disorders or myelodysplastic syndromes (MDSs).3 Moreover, conventional antitumor treatments make a selective pressure for p53-inactivated tumor cells, and the development of drug resistance remains a serious problem. In this context, a special effort is currently dedicated to targeted therapies whose cornerstone—at least in terms of clinical success—have been imatinib for chronic myelogenous leukemia,4 while several other one component of more complex antitumor therapeutic regimens.

Emerging evidence, however, suggests that TRAIL acts synergistically with chemotherapy and radiotherapy, combinations of these with TRAIL or with cytokines that mobilize effector cells equipped with membrane-bound TRAIL (like natural killer [NK] cells, T cells, monocytes, and neutrophils) might prove effective in patients with cancer.5-15 A deeper understanding of the mechanisms of synergy between TRAIL and other antitumor drugs and biological agents is therefore worthy of further scientific effort. In this regard, in a recent paper, Carter et al16 demonstrated that triptolide, a diterpenoid compound, was able to sensitize AML cells to TRAIL–induced apoptosis via a decrease of antiapoptotic XIAP and a p53-mediated increase of TRAIL-R2. However, although TRAIL-R1 and TRAIL-R2 are commonly referred to as the “death receptors” for their well-characterized activity of transducing the apoptotic signal, mounting experimental evidence suggests that they are also involved in nonapoptotic functions. In fact, TRAIL has been recently described as a promoter/inhibitor of the maturation of erythrocytes, megakaryocytes, and monocytes.17-21

It is well established that several isoforms of PKC have a role in the regulation of TRAIL activity.19,21-23 In general, PKC isoenzymes play central roles in various cellular signaling pathways, participating in a variety of protein phosphorylation cascades that regulate/modulate cellular structure and gene expression.24-29 Specifically, we found that PKC\(\varepsilon\) protects late erythroid precursors from the apoptogenic effects of TRAIL, to which are—on the contrary—exposed in their earlier phases of differentiation.19 In parallel, we also demonstrated that the timing and expression levels of PKC\(\varepsilon\) act as regulators of the megakaryocytic differentiation of human CD34 cells.21
Table 1. Clinical characteristics of patients with AML

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sample source</th>
<th>Blasts, %</th>
<th>CD33, %</th>
<th>CD34, %</th>
<th>FAB</th>
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<tr>
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<td>99</td>
<td>85</td>
<td>M4</td>
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<td>BM</td>
<td>85</td>
<td>99</td>
<td>99</td>
<td>M1</td>
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<tr>
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<td>PB</td>
<td>90</td>
<td>97</td>
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<td>BM</td>
<td>95</td>
<td>92</td>
<td>0</td>
<td>M5</td>
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</tbody>
</table>

The CD33 and CD34 surface expression was studied by flow cytometry gating on the blast population. The AML classification was made according to the French-American-British classification (FAB).

A variety of compounds with the capacity to modulate PKC activity have been studied.23,24 These differ in selectivity, potency, and isoenzyme preference. Two such agents, bryostatin 1 and phorbol esters, are being evaluated in clinical trials.30-35 Several phorbol ester–induced effects are mediated by the temporal activation, translocation, and/or suppression of selected PKC isoforms.36-38 In general, acute exposure to phorbol esters activates different PKC isoforms, while chronic exposure down-regulates the activity of these enzymes.36,37,39,40 Phorbol esters induce a broad range of cellular effects, including maturation/differentiation of hematopoietic cell lines41,42 and apoptosis in prostate cancer cells.43 In a preclinical study on prostate cancer cells, phorbol esters (TPA) and paclitaxel in combination had a stronger inhibitory effect on the growth of tumor cells both in vitro and in vivo, suggesting the possibility of using TPA alone or in combination with paclitaxel in patients with prostate cancer.44 Moreover, phorbol esters are also administered at very low concentrations in patients with myeloid leukemias.31,34,35 On the contrary, in a phase 1 clinical trial with escalating doses of phorbol esters in patients with relapsed or refractory malignancies, serious adverse events were described at high doses of administration.35

Given this complex background, we studied here the effects of TRAIL in association with the phorbol ester 4-β-phorbol-12,13-dibutyrate (PDBu) on primary AML cells. We found that PDBu sensitizes AML cells to both the apopoticogenic and the prodifferentiative effects of TRAIL by modulating intracellular PKCe levels and the molecular signaling machinery downstream apoptogenic TRAIL receptors, without affecting surface TRAIL receptors expression.

CD41, CD61, and CD42b cell lineages. TF-1 cells also express CD34 and are cytokine-dependent for their growth, with a more immature phenotype than HeL and K562 cells.

TF-1, HeL, and K562 cells were grown in 10% FBS-enriched RPMI medium at the optimal density of 0.5 × 10^6 cells/mL. TF-1 cells were maintained in the presence of 3 ng/mL IL-3 (PeproTech, London, United Kingdom). TF-1, HeL, and K562 cells were treated with the phorbol ester PDBu (TF-1: 5 nM; HeL and K562: 20 nM) to induce MK differentiation in the presence or absence of 25 ng/mL recombinant TRAIL (Alexis Biochemical, San Diego, CA).

Cell morphology was analyzed by light microscopy at day 3 of culture. Aliquots of cultured cells were centrifuged with a StatSpin CytoFuge (StatSpin, Norwood, MA) at 20g for 4 minutes. Slides were stained with Giemsa (Sigma-Aldrich) and examined by an Eclipse 80i digital light microscope (Nikon, Tokyo, Japan). The images were captured using the ACT-2U software (Nikon).

Flow cytometric analysis

Aliquots of 0.3 × 10^6 cells/experimental point were labeled by a panel of anti–TRAIL-Rs mAbs (Alexis Biochemical). Expression of TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 was analyzed by indirect staining with 1 μg HS101 anti–human TRAIL-R1, HS201 anti–human TRAIL-R2, HS301 anti–human TRAIL-R3, and HS401 anti–human TRAIL-R4 mAbs, followed by R-phycocerythrin (RPE)–conjugated goat anti–mouse IgG (Immunotech, Luminy, France) as a second reagent.

To follow megakaryocytic differentiation, aliquots of 0.3 × 10^6 cells/experimental point were labeled with RPE-conjugated anti-CD61 (Pharningen/Becton Dickinson, San Jose, CA), RPE-conjugated anti-CD41 (Chemicon, Temecula, CA), cytin-5 (Cy5)–conjugated anti-CD42b (Pharmingen/Becton Dickinson), RPE-conjugated anti-CD34 (Immunotech), RPE-conjugated anti-CD33 (Pharningen/Becton Dickinson), RPE-conjugated anti-CD14 (Chemicon), and RPE-conjugated anti–glycophorin A (DAKO, Glostrup, Denmark). Working dilutions of all reagents were previously optimized by serial dilution tests. Analysis of the samples was performed by an Epics XL flow cytometer (Beckman Coulter, Fullerton, CA) and the Expo ADC software (Beckman Coulter). The instrument was calibrated daily with a set of standardized beads (DAKO). These consisted of a set of beads each with a different known amount of either FITC, PE, or Cy5 expressed in units of MESF (molecules of equivalent soluble fluorescein). Thus, a standard curve was constructed each day by plotting MESF values for the beads against the median channel in which the peak was displayed.45

To quantify the platelet production in culture, fixed volumes of the culture supernatants were collected, incubated with anti-CD41–RPE and calcine AM (Sigma-Aldrich; to exclude fragments) and added to a fixed volume of calibration beads (DAKO) at known concentration. Both the platelet and beads populations were simultaneously identified in flow cytometry on the forward scatter (FS) versus logarithmic side scatter (SS) dot plot. The absolute platelets count was performed on the gated CD41+/calcine AM+ platelet population.

PKCe overexpression, siRNA design, and transfection

The green fluorescent protein (GFP)–PKCe expression and control plasmid were kindly provided by Prof Peter Parker (Cancer Research UK, London Research Institute, London, United Kingdom). The murine GFP-tagged PKCe and the murine GFP-K525M mutated PKCe constructs were cloned in the pCDNA3.1 hygro vector fused with GFP.46

PKCe expression levels were down-regulated in primary AML cells by transfection of double-stranded siRNAs (dsRNA) designed to target PKCe sequences corresponding to nt 233 to 244, 429 to 450, 942 to 963, and 1158 to 1179 on human PKCe mRNA (NM005400). The target sequences were as follows: 5’-aagat cgagc tggctg-3’, 5’-aagat ccagc ttgctg-3’, 5’-aacta caagg tccct acctc-3’, and 5’-aaaca gttc ggctg-3’. The respective sense and antisense RNA sequences were synthesized by the Silencer siRNA Construction Kit (Ambion, Austin, TX).47 Non-specific siRNA duplexes containing the same nucleotides, but in irregular sequence (ie, scrambled PKCe siRNA), were prepared according to the manufacturer’s protocol and used as controls.

Methods

Cell cultures and treatment

Approval for this study was obtained from the Institutional Review Boards of the Department of Anatomy, Pharmacology and Forensic Medicine and of the Department of Clinical Sciences at the University of Parma (Parma, Italy).

Peripheral blood (PB) cells from 11 patients with acute myeloid leukemia (AML) were collected after informed consent was obtained in accordance with the Declaration of Helsinki. Aliquots were immediately used for flow cytometry phenotyping (Table 1). Cells were grown in 20% fetal bovine serum (FBS)–enriched RPMI medium, at the density of 2 × 10^6 cells/mL, in the presence of 20 nM PDBu (Sigma-Aldrich, St Louis, MO) to induce megakaryocyte (MK) differentiation, in the presence or absence of 25 ng/mL recombinant TRAIL.

TF-1, HeL, and K562 cell lines express surface antigens specific for both the erythroid (glycophorin; CD71) and the megakaryocytic (MK,
To maximize transfection efficiency, GFP-PKCe plasmids (1 µg/transfection) and PKCe siRNAs (100 nM each) were delivered using the Amaxa nucleofection technology (Amaxa, Koeln, Germany) according to the manufacturer’s protocols.47

Western blot

Cultured cells were counted and 2 × 10⁶ cells were collected at specific time points, washed in phosphate-buffered saline (PBS), and centrifuged at 200g for 10 minutes. Pellets were resuspended in a cell lysis buffer (50 mM Tris·HCl [pH 7.4], 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSE, 1 mM Na₃VO₄, and 1 mM NaF) supplemented with fresh protease inhibitors, and protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL). A total of 30 µg of proteins from each sample were then migrated in 5% SDS-acrylamide gels and blotted onto nitrocellulose filters.

Blotted filters were blocked and incubated with specific primary antibodies diluted as described in the manufacturers’ protocols. Specifically, rabbit polyclonal anti-PKCe (Upstate, Lake Placid, NY) and anti-caspase-3 (Cell Signaling, Lake Placid, NY) antibodies were used at the concentration of 1 µg/mL and at a dilution of 1:500, respectively. The mAbs anti-caspase-8 (Alexis Biochemical) and anti-FLIP long (Alexis Biochemicals) were diluted 1:500. The mAb anti-β-actin (Sigma-Aldrich) was diluted 1:5000.

Filters were washed and further incubated for 1.5 hours at room temperature with 1:5000 peroxidase-conjugated anti-rabbit or with 1:2000 peroxidase-conjugated anti-mouse IgG (Pierce) in the primary antibody working solution at room temperature. Specific reactions were revealed with the ECL Supersignal West Pico Chemiluminescent Substrate detection system (Pierce).

Assessment of apoptosis

Cell culture viability was assessed by trypan blue exclusion. Apoptotic cells were identified by flow cytometry either as subdiploid peaks generated by DNA fragmentation or by annexin V/propidium iodide (PI) staining. Specifically, cells identified by flow cytometry either as subdiploid peaks generated by DNA fragmentation or as annexin V/propidium iodide (PI) staining. Specifically, cells identified by flow cytometry either as subdiploid peaks generated by DNA fragmentation or as annexin V/propidium iodide (PI) staining. Specifically, cells identified by flow cytometry either as subdiploid peaks generated by DNA fragmentation or as annexin V/propidium iodide (PI) staining.
TF-1 cells showed the highest expression of CD41 and CD61 at 5 nM PDBu for 72 hours; PDBu induces cell death at higher concentrations, while HeL and K562 showed the best response at 20 nM for 72 hours. Glycophorin expression decreased in parallel, as expected (Figure 2B). The large standard deviations observed with high doses of PDBu were due to its relative toxicity, and therefore the lowest concentrations able to increase CD41 and CD61 cells surface expression were chosen. At these concentrations, PDBu induced a variable degree of cell death that, however, did not exceed 40% (Figure 2C). Primary AML cells were induced to the maximal expression of CD41 by 20 nM PDBu for 72 hours with a variable induction of cell death as well (Figure 2D,E).

**PDBu sensitizes AML cells to the effects of TRAIL**

We then added TRAIL to PDBu-treated AML cells and cell lines. Since it is known that TRAIL can exert both apoptogenic and prodifferentiative effects, we monitored again both phenotypical antigen expression and cell viability.

Figure 3A shows the effects of 25 ng/mL of TRAIL on the PDBu (5 nM)--induced progression of MK differentiation of TF-1 cells. The association of TRAIL to PDBu significantly boosts the expression of MK lineage-related antigens, while glycophorin A expression virtually disappears. Moreover, given that TRAIL, per se is apoptogenic in the cell lines, we were not surprised to observe a synergistic effect of TRAIL and PDBu in the induction of cell death, as revealed by the CI calculation at ED50, ED75, and ED90 (Figure 3B).

More importantly, PDBu sensitizes primary AML cells to TRAIL, promoting both cell differentiation and cell death. The combined treatment of AML cells with PDBu and TRAIL in fact induced a decrease of CD34 and CD33 surface antigen expression, which was accompanied by the increase of CD41 expression, both in terms of percentage of positive cells (Figure 3C bottom panels) and absolute numbers of antigens expressed/cell (Figure 3C top panels). On the contrary, there was no significant modulation of the CD14 expression.

MK differentiation was also studied by morphology and platelet production. Primary AML cells treated with PDBu and TRAIL became larger and multinucleated (Figure 3D) and were able to release increased number of platelets after 7 days of culture (Figure 3E). Moreover, although TRAIL was not cytotoxic to primary AML cells, the association of TRAIL to PDBu significantly enhanced AML cell death (Figure 3F,G).

**PDBu down-modulates PKCe expression in leukemic cells**

Since we have previously demonstrated that the effects of TRAIL in hematopoietic cells largely depend on PKCe expression levels, with high PKCe levels protective against TRAIL-induced apoptosis, we asked if PDBu could induce a specific modulation of PKCe expression.

We therefore assayed the levels of PKCe upon PDBu-induced MK differentiation in the presence or absence of 25 ng/mL TRAIL.

As shown in Figure 4, both primary AML cells and cell lines constitutively express PKCe at various levels. However, when the cells are treated with PDBu, the PKCe levels invariably decrease. TRAIL alone is also able to reduce PKCe protein levels, and the association of TRAIL to PDBu always potentiates the reduction (Figure 4B,D).

**PDBu-mediated down-modulation of PKCe sensitizes leukemic cells to TRAIL**

To formally prove that PDBu-mediated down-modulation of PKCe was causal to the sensitization of leukemic cells to TRAIL, we (1) overexpressed PKCe or an inactive PKCe K522M mutated (PKCem) kinase...
The combination index (CI) was expressed as the average of the CI values obtained at the ED50, ED75, and ED90 and indicates synergism between TRAIL and PDBu in the staining; original magnification, cultured in the presence of TRAIL plus PDBu. Cells progressively acquired an enlarged and multinucleated morphology, typical of differentiating megakaryocytes (Giemsa doses of PDBu ()), or both agents ()), TRAIL (P), and PI staining. Data from 3 independent experiments are expressed as means plus or minus SD. The combination index (CI) was expressed as the average of the CI values obtained at the ED_{50}, ED_{75}, and ED_{90} and indicates synergism between TRAIL and PDBu in the induction of cell death. (C) Expression of CD41, CD14, CD33, and CD34 antigens on the surface of AML cells cultured with or without 20 nM PDBu in the presence or absence of 25 ng/mL TRAIL for 3 days. Top panels show the absolute numbers of surface antigens expressed per cell (MESF). Bottom panels show the percentage of positive cells. Tendency lines are calculated as means plus or minus SD. *P < 0.05 versus control; #P < 0.05 TRAIL plus PDBu versus PDBu alone. (D) Light microscopy of primary AML cells cultured in the presence of TRAIL plus PDBu. Cells progressively acquired an enlarged and multinucleated morphology, typical of differentiating megakaryocytes (Giemsa staining; original magnification, ×40; insets, ×100). (E) Relative number of platelets in the culture of AML cells at day 7 of treatment with TRAIL, PDBu, or their combination. The number of platelets was calculated by flow cytometry on the gated CD41+/calcium AM+ population analyzed in combination with a known number of calibration beads. Data from 3 independent experiments are expressed as means ± SD. *P < 0.05 versus control; #P < 0.05 TRAIL plus PDBu versus PDBu alone. (F) Apoptosis of AML cells treated for 3 days with or without 20 nM PDBu in the presence or absence of 25 ng/mL TRAIL. Cell viability is shown as percentage of the control (DMSO). Data from 11 patients with AML are expressed as means plus or minus SD. *P < 0.05 versus control; #P < 0.05 TRAIL plus PDBu versus PDBu alone. (G) Cell viability of AML cells cultured with or without 20 nM PDBu in the presence or absence of 25 ng/mL TRAIL. A representative flow cytometric detection of PI incorporation by death cells is shown.

First, PDBu treatment does not modify the surface expression of TRAIL receptors (Figure 6A). Subsequently, we explored the levels of full-length (inactive) caspase-8, FLIPL, and caspase-3, in primary AML cells cultured with PDBu in the presence or absence of 25 ng/mL TRAIL. Primary AML cells expressed high levels of full-length caspase-8, a variable level of FLIPL, and low levels of caspase-3 (Figure 6B). Collectively, the data reported in Figure 6B show that (1) PDBu induces a decrease of FLIPL levels, thus predisposing leukemic cells to the apoptogenic effect of TRAIL; and (2) the addition of TRAIL to PDBu induces an activation of the extrinsic apoptotic pathway, as indicated by the marked decrease of procaspase-8 (seemingly due to its cleavage, and thus to its activation) and the clear increase of the cleaved (active) form of caspase-3.

Discussion

Besides its well-known apoptogenic effects, TRAIL signaling does not only lead to the activation of effector caspases and apoptosis, but can also induce nonapoptotic pathways, including the activation of mitogen-activated protein (MAP) kinases and NF-kB, leading to cell proliferation and differentiation. The differential molecular mechanism(s) underlying the apoptogenic versus the differentiative/proliferative effects of TRAIL are not yet clear, although the NF-kB pathway appears to be relevant. However,
The expression of antiapoptotic Bcl-2 and Bcl-xL proteins is regulated by PKCε levels in hematopoietic cells, and these proteins have proven to be effective in preventing TRAIL-induced apoptosis in hematopoietic progenitors. Since (1) induction of differentiation and induction of apoptosis are both useful strategies against cancer in general and leukemia in particular; (2) AML cells are generally resistant to the apoptogenic effects of TRAIL; (3) PKCε levels modulate the apoptogenic response of hematopoietic progenitors to TRAIL; and (4) phorbol esters, which induce the translocation of PKC isoforms from the cytosolic to the particulate fraction controlling their access to substrates, induce the maturation of hematopoietic cell lines, and are now used in phase 1 clinical trials against myeloid leukemia, we decided to test the hypothesis that phorbol esters could sensitize

Figure 4. TRAIL and PDBu down-modulate PKCε. (A) Detection of endogenous PKCε protein in AML cells by Western blot. PKCε was revealed by the specific antibody. β-actin was monitored for protein loading. AML cells were treated for 3 days with DMSO (controls), PDBu (20 nM), and TRAIL (25 ng/mL), alone or in combination. PDBu, TRAIL, and, more evidently, their combination, induce a decrease of PKCε. A representative experiment is shown. (B) Levels of PKCε protein expression in AML cells treated for 3 days with DMSO (controls), PDBu (20 nM), and TRAIL (25 ng/mL), alone or in combination. Densitometric measurements of Western blots from 6 AML samples (means ± SD). * P < .05 versus control; # P < .05 TRAIL plus PDBu versus PDBu alone. (C) Detection of endogenous PKCε protein in TF-1, HeL, and K562 cells by Western blot. β-actin was monitored for protein loading. TF-1, HeL, and K562 cells were treated for 3 days with DMSO (controls), PDBu (TF-1, 5 nM; HeL and K562, 20 nM), and TRAIL (25 ng/mL), alone or in combination. Although the different cell lines show different basal levels of PKCε expression, PDBu, TRAIL, and, more evidently, their combination, invariably induce a decrease of PKCε. (D) Levels of PKCε proteins expression in TF-1, HeL, and K562 cells treated for 3 days with DMSO (controls), PDBu (TF-1, 5 nM; HeL and K562, 20 nM), and TRAIL (25 ng/mL), alone or in combination. Densitometric measurements of Western blots from 5 independent experiments (means ± SD). * P < .05 versus control; # P < .05 TRAIL plus PDBu versus PDBu alone.

Figure 5. PKCε overexpression or down-modulation. (A) Western blot of PKCε in TF-1 cells overexpressing PKCε (PKCε) or mutated PKCε (PKCε-GFP) and endogenous PKCε (PKCε) proteins are shown in the immunoblot. (B) Cell viability of TF-1 cells overexpressing PKCε or PKCε-GFP. After 24 hours, the cells were treated with or without 5 nM PDBu in the presence or absence of 25 ng/mL TRAIL for 3 days. Data from 3 experiments are expressed as means plus or minus SD. * P < .05 versus control; # P < .05 TRAIL plus PDBu versus PDBu alone. (C) PKCε overexpression reduces the density of surface MK markers in TF-1 cells. TF-1 cells were transfected with PKCε (black histograms, PKCε) or mutated PKCε (gray histograms, PKCε) after 24 hours, the cells were cultured with or without 5 nM PDBu and analyzed 3 days later. Data from 3 experiments are expressed as means plus or minus SD of absolute numbers of surface antigens (MESF) expressed per cell. * P < .05 versus control; # P < .05 PKCε versus PKCε-GFP. (D) Western blot of PKCε in primary AML cells transfected with PKCε-specific siRNA (PKCε siRNA) or control siRNA (Scrambled siRNA). (E) Cell viability of primary AML cells transfected with PKCε-siRNA or control siRNA (Scrambled siRNA). After 48 hours, the cells were treated with or without 20 nM PDBu in the presence of 500 ng/mL TRAIL for 3 days. Data from 3 experiments are expressed as means plus or minus SD. * P < .05 versus control; # P < .05 PKCε siRNA versus Scrambled siRNA.
AML cells to the effects of TRAIL, an effect that could have an impact on the therapeutic strategies for AML.

Notwithstanding the expression of TRAIL-R2, primary AML cells are resistant to TRAIL, while TRAIL is apoptogenic for the TF-1, K562, and HeL cell lines. Treatment of primary AML cells with 20 nM PDBu for 72 hours induced both cytotoxicity and cell differentiation along the MK lineage. The addition of 25 ng/mL TRAIL to PDBu-treated AML cells synergistically enhanced both cytotoxicity and cell differentiation. Searching for the molecular basis of the sensitization to TRAIL, we immediately focused on PKCε, since this isoform of PKC has a key role in the modulation of the effects of TRAIL in normal hematopoiesis. One could imagine that, because phorbol esters are general activators of PKC, induction of terminal MK differentiation of leukemic cells by PDBu would keep PKCε at high levels. On the contrary, PKCε is down-modulated during PDBu-induced MK differentiation of both primary AML cells and leukemic cell lines, similarly to what happens in normal, TPO-induced, MK differentiation of primary CD34 progenitors.

Overexpression of PKCε—but not of a catalytically inactive, mutated form of PKCε—abolishes both the cytotoxic and the prodifferentiative effects of TRAIL on PDBu-treated leukemic cells. On the contrary, the down-modulation of PKCε levels in AML cells by PKCε-specific siRNA enhances both the cytotoxic and the prodifferentiative effects of the association of PDBu and TRAIL. Although other compounds known to sensitize tumor cells to TRAIL, such as the recently described triptolide,16 enhance death receptor expression, this was not the case for PDBu, which did not modify the phenotypical expression of TRAIL-Rs. However, several key molecules of the TRAIL-Rs-dependent apoptotic signaling are modulated by the association of TRAIL to PDBu in AML cells. Specifically, the association of TRAIL and PDBu definitely activate caspase-8 and caspase-3 while decreasing FLIP levels, thus opening the way for a fully apoptogenic signaling in AML cells. Specifically, our results suggest that PDBu predisposes leukemic cells to the apoptogenic effects of TRAIL through a decrease of FLIPL. TRAIL, in conclusion, holds the intuitive advantage of being both cytotoxic and—at the same time—prodifferentiative for the PDBu-treated leukemic cell population. AMLs are characterized by the predominance of immature cells, mainly blasts. Our results suggest that PDBu primarily suppresses the protection of AML blasts from the action of apoptogenic death ligands. In parallel, given the role of PKCs—and specifically PKCε—in the MK differentiation, PDBu theoretically creates biologically favorable conditions to allow terminal differentiation of surviving blasts. One limitation of this study is the relative small number of patients studied. Given the differentiative heterogeneity of AML (Table 1), it is, however, tempting to speculate that the combination of PDBu and TRAIL preferentially induces cell death of more immature phenotypes while promoting terminal differentiation of the more mature ones.

Our data are limited to the induction of megakaryocytic differentiation. The analogy of the effects of TRAIL with the normal MK development raises the question of whether this double-faceted effect of TRAIL would also take place when inducing cell differentiation along lineages other than MK. However, it must be noted that a similar prodifferentiative effect of TRAIL has been described in HL-60 cells,17 while ATRA has been demonstrated to induce apoptosis of differentiating promyelocytic cells via a paracrine induction of TRAIL.51

Overall, our data suggest that using TRAIL in combination with phorbol esters (or possibly other, more specific, PKCε modulators) could represent a useful strategy to induce both apoptosis and/or terminal differentiation of AML cells.

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Authorship

Contribution: G.G. designed and performed research and analyzed data; P.M. designed research and analyzed data; C.C., C. Micheloni, and C. Malinverno performed research; P.L. contributed tools; A.B. analyzed data; and M.V. designed research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
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Phorbol ester–induced PKCδ down-modulation sensitizes AML cells to TRAIL-induced apoptosis and cell differentiation

Giuliana Gobbi, Prisco Mirandola, Cecilia Carubbi, Cristina Micheloni, Chiara Malinverno, Paolo Lunghi, Antonio Bonati and Marco Vitale