Role of protein kinase C ζ isoform in Fas resistance of immature myeloid KG1a leukemic cells

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Leukemic CD34+ immature acute myeloid leukemia (AML) cells express Fas receptor but are frequently resistant to Fas agonistic reagents. Fas plays an important role in T-cell–mediated cytoxicity, and recently it has been suggested that altered Fas signaling may contribute to drug resistance. Therefore, Fas resistance could be one of the mechanisms by which AML progenitors escape chemotherapy or T-cell–based immune intervention. However, the molecular mechanism of Fas resistance in AML cells has not been identified. Fas signaling can be interrupted at 3 main levels: Fas clustering, alteration of death-inducing-signaling-complex (DISC) formation, and effector caspase inhibition of downstream caspase-8. This study shows that in the Fas-resistant CD34+CD38− KG1a cells, Fas agonists resulted in Fas aggregation but not in caspase-8 activation, related to a defect in DISC formation. However, pre-treatment with chelerythrin, but not with calphostin C, resulted in the restoration of Fas-induced caspase-8 activation and cytoxicity, suggesting that some atypical protein kinase C (PKC) isoforms contributed to the lack of DISC formation. Indeed, treatment with antisense oligonucleotides directed against PKCζ and enforced expression of Par-4, a negative regulator of PKCζ activity, restored Fas-induced caspase-8 activity and apoptosis. Moreover, it was found that PKCζ interacts with FADD and that PKCζ immunoprecipitates prepared from KG1a cells are able to phosphorylate FADD in vitro, whereas this phosphorylation is dramatically reduced in Par-4 transfected cells. In conclusion, it is suggested that in AML cells, PKCζ plays an important role in Fas resistance by inhibiting DISC formation, possibly by phosphorylating FADD.

Introduction

Fas (APO-1/CD95) is a 45-kd membrane protein that belongs to the tumor necrosis factor (TNF)–nerve growth factor receptor family, a group of type 1 transmembrane receptors.1 Mutational analysis of Fas and the human TNF receptor (TNFR-1) proteins demonstrates that the cytoplasmic domains share a homologous region necessary to transduce the apoptotic signal. This conserved region of approximately 70 amino acids was, therefore, designated as the death domain (DD). The only known physiological ligand of Fas, Fas-L, belongs to the family of TNF-related cytokines.2 Fas-L is synthesized as a transmembrane molecule, and soluble Fas-L trimer can be generated through processing by a metalloprotease.3,4 Engagement of Fas by agonistic anti-Fas antibodies or by Fas-L triggers apoptosis in a variety of cell types. However, only membrane-bound or multimerized Fas-L induces cell death.3,4 Moreover, ligand-dependent activation of Fas death pathway requires the oligomerization of Fas receptor, but ligand-independent activation can occur on Fas aggregation induced by Fas overexpression or treatment with anticancer drugs or radiation.5,9 Clustering of Fas recruits Fas-associated death domain (FADD)–containing protein, which is a bipartite molecule with a death effector domain (DED) at the amino terminus and a DD at the carboxyl terminus. FADD binds to Fas through a DD–DD interaction and recruits the DED-containing procaspase-8 through a DED–DED interaction. The formation of this death-inducing signaling complex (DISC) results in caspase-8 activation, believed to be the first step of a proteolytic cascade that triggers the activation of other caspases such as caspase-3, -7, and -6.10,11 Although other cell death pathways could be initiated from Fas activation,12-14 analysis of lymphocytes from FADD−/− mice has demonstrated the prominent role of the FADD/caspase-8 pathway in Fas-mediated cell death.15 Normal CD34+ hematopoietic cells, including the most immature CD34+CD38− subset, express Fas at a low level and are resistant to Fas-induced apoptosis unless they are treated with TNF-α or interferon (IFN)-γ.16-19 These studies have shown also that these cytokines enhanced Fas expression in CD34+ cells; however, they did not provide direct evidence that TNF-α or IFN-γ–induced sensitization to Fas-induced apoptosis was attributed to increased Fas expression. Moreover, when cultured in the presence of hematopoietic growth factors, CD34+ cells expressed functional Fas; indeed, the CD34+ Fas+ cell population gradually lost CD34 expression and shifted to a CD34− Fas+ and Fas-sensitive cell population.18 These results suggest that Fas is expressed as part of a differentiation program of hematopoietic cells; in fact, functional Fas is expressed in terminally differentiated myeloid cells, including neutrophils, eosinophils, and monocytes.20,21 Fas distribution and function appear to be not very different in leukemic myelopoiesis. Indeed, most fresh CD34+ acute myeloid leukemia (AML) cells express Fas, whereas they are frequently resistant to Fas-induced apoptosis.22,23 These results

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Supported by the Association pour la Recherche contre le Cancer (grants 5526 and 5968). A.d.T. is the recipient of a grant from the Ministère de l’Education Nationale, de l’Enseignement Supérieur, et de la Recherche.

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suggest that in AML cells, as in early normal progenitors, potent negative regulators interfere either upstream or downstream of DISC formation. This may have important physiopathologic and therapeutic implications in AML. First, based on a recent study suggesting that the Fas–Fas-L system exerts a negative regulatory effect on committed progenitor expansion, it may be that Fas resistance contributes to leukemic clone expansion. Second, because the Fas–Fas-L system plays an important role in T-cell–mediated cytoxicity, it is conceivable that resistance to Fas decreases the efficiency of graft-versus-leukemic reaction after allogeneic bone marrow transplantation or donor lymphocyte infusion. Third, because it has been reported that the ligand-decreases the efficiency of graft-versus-leukemic reaction after resistance contributes to leukemic clone expansion. Second, directed against PKC (Goettingen, Germany; product number 01669). Other products were rected against PKC Perray-en-Yvelines, France). Sense and antisense oligonucleotides di- anti–Par-4 were purchased from Santa Cruz Biotechnology (TEBU, Leersheim, France); anti-FADD monoclonal antibodies were purchased from Euromedex (Souffelweyersheim, France), anti-actin monoclonal anti- Fontana, Lausanne, Switzerland). Anti-PKC was incubated with caspase-8 colorimetric substrate for 2 hours at 37°C. Fas-L-FLAG was purchased from Alexis (Coger, France). Recombinant Fas-L–FLAG (Alexis, San Diego, CA) and 1 μM anti-FLAG monoclonal antibody (Sigma, Saint-Quentin-Fallavier, France) for 15 minutes or 1 hour. Cells were then centrifuged and lysed in lysis buffer (0.2% NP-40, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM sodium vanadate, 10% glycerol, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride [PMSF]) for 20 minutes on ice, followed by centrifugation at 10 000g for 15 minutes. Protein concentration in the supernatants was determined as previously described. For each lysate, 40 μg total protein was boiled for 5 minutes at 95°C in the presence of 3% β-mercaptoethanol. Proteins were separated on 12.5% (wt/vol) SDS–polyacrylamide gel electrophoresis (PAGE) and were transferred electrophoretically onto nylon membranes (Hybond-C extra; Amersham Life Science, Cergy-Pontoise, France). Nonspecific binding sites were blocked in 10 mM Tris-buffered saline containing 0.1% Tween-20 and 10% nonfat milk. Membranes were then incubated overnight at 4°C with specific primary monoclonal antibody diluted at an appropriate concentration in 10 mM Tris-buffered saline containing 0.1% Tween-20 and 1% nonfat milk. Membranes were then washed 5 times at room temperature, and bound immunoglobulin was detected with anti-isotype monoclonal antibody coupled to horseradish peroxidase (Beckman-Coulter). The signal was visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, United Kingdom) and autoradiography.

**DISC formation analysis**

Exponentially growing cells (100 × 10⁶) were incubated with 1 μg/mL Fas-L–FLAG (Alexis, San Diego, CA) and 1 μg anti-FLAG monoclonal antibody (Sigma, Saint-Quentin-Fallavier, France) for 15 minutes or 1 hour. Cells were then centrifuged and lysed in lysis buffer (0.2% NP-40, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM sodium vanadate, 10% glycerol, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.1 mM PMSF) before protein A–Sepharose was added. Immunoprecipitates were washed 3 times in lysis buffer without protease inhibitors before SDS-PAGE and Western blot analysis.

**Caspase-8 activity assay**

Caspase-8 colorimetric activity assay (R&D Systems, Abingdon, United Kingdom) was performed according to the manufacturer’s recommendation. Briefly, exponentially growing cells treated by CH11 monoclonal antibody (2 μg/mL) for 4 hours were collected by centrifugation. Lysis buffer was added on the cell pellet, incubated on ice for 10 minutes, and centrifuged at 10 000g for 1 minute. For each lysate, 100 μg total protein was incubated with caspase-8 colorimetric substrate for 2 hours at 37°C. Cleavage of the substrate by caspase-8 was quantitated spectrophotometrically at a wavelength of 405 nm.

**PKCζ antisense experiments**

Blocking experiments were performed with antisense or sense phosphorothioate oligonucleotides (10 μM) directed against PKCζ. Briefly, exponentially growing cells were cultured with sense or antisense oligonucleotides
Cell lysates (10^6) were prepared in lysis buffer for 30 minutes on ice, sonicated, and centrifuged (15 minutes, 10,000g at 4°C). Supernatants were normalized for protein concentration, and each sample (1mg protein) was immunoprecipitated with anti-PKCζ monoclonal antibody (4μg) or anti-FADD monoclonal antibody (2μg) and collected by absorption to protein G-Sepharose. Immunoprecipitates were washed 3 times in RIPA buffer without protease inhibitors before analysis by SDS-PAGE and Western blotting.

In vitro PKCζ kinase assay

Cell lysates (5 × 10^6) were prepared in RIPA lysis buffer for 30 minutes on ice, sonicated, and centrifuged (15 minutes, 10,000g at 4°C). Supernatants were normalized for protein concentration, and each sample (1mg protein) was immunoprecipitated with anti-PKCζ monoclonal antibody (4μg) or anti-FADD monoclonal antibody (2μg) and collected by absorption to protein G-Sepharose. Immunoprecipitates were washed 3 times in RIPA buffer without protease inhibitors before analysis by SDS-PAGE and Western blotting.

Immunoprecipitation

Cell lysates (10 × 10^6) were prepared in lysis buffer (20 mM HEPES, 2 mM EDTA, 125 mM NaCl, 0.1% NP40, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 0.5 mg/mL benzamidine, 1 mM PMSF, 1 mM DTT) for 30 minutes on ice followed by centrifugation (3 minutes, 14,000g). Supernatants were normalized for protein concentration, and each sample (1mg protein) was immunoprecipitated with anti-PKCζ monoclonal antibody and collected by absorption to protein G-Sepharose. Immunocomplexes bound to protein G-Sepharose were washed in lysis buffer without PMSF and subsequently were resuspended in reaction buffer (20 mM HEPES, 1 mM DTT, 10 mM MgCl2, 4 μg/mL phosphatidylserine, 20 μM cold ATP). For each sample, 10 μCl [γ-32P] ATP (6000 Ci/mmoll [222 TBq/mmoll]; ICN, Orsay, France) and 1 μg FADD agarose or 3 μg histone H1 were added. Samples were then incubated for 5 minutes at 32°C. The reaction was terminated by the addition of protein loading buffer. Proteins were separated on 10% SDS-PAGE, and the gel was subjected to autoradiography. In parallel, an aliquot of each sample was analyzed by Western blot using anti-PKCζ monoclonal antibody to quantify immunoprecipitated proteins.

Par-4 transfection in KG1a cells

Exponentially growing cells were transfected by a plasmid containing full-length Par-4 cDNA sequence (kindly gift from M. T. Diaz-Meco, Madrid, Spain) using Effectene transfection reagent (Qiagen, Courtaboeuf, France) according to the manufacturer’s instructions. Clones were further selected for Par-4, CD34+, CD38− expression by Western blot analysis or flow cytometry.

Statistics

Quantitative experiments were analyzed using Student t test. All P values resulted from the use of 2-sided tests.

Results

Fas expression and function in KG1a cells

KG1a, U937, and Jurkat cells were treated with increasing concentrations of CH11 monoclonal antibody for 30 minutes in cold medium, then were stained by an indirect immunofluorescence technique using phycoerythrin-labeled goat–anti-mouse immunoglobulin (IgM) antibody. Fluorescence was evaluated by flow cytometry for each cell line. Saturating concentrations ranged between 0.5 and 2 μg/mL, depending on the cellular model. At the saturating dose of 2 μg/mL, KG1a cells displayed mean fluorescence intensity similar to, if not higher than, that of Fas-sensitive Jurkat or U937 cells (data not shown).

KG1a cells were then treated with various doses of CH11 (2-10 μg/mL) in supplemented Iscoves modified Dulbecco medium culture for 24, 48, and 72 hours. Cell viability was measured by Trypan blue dye exclusion assay. CH11 monoclonal antibody, at a 2 μg/mL dose, induced only a modest, though significant, growth inhibitory effect on KG1a cells compared to IgM-isotypic control-treated cells (Figure 1A). Higher doses (up to 10 μg/mL) were also inefficient for inducing KG1a cell death (data not shown). However, CH11-treated Jurkat and U937 cells rapidly died, the former more sensitive than the latter (Figure 1B). Morphologic examination after DAPI staining showed typical features of apoptosis in CH11-treated Jurkat and U937 cells, whereas CH11-treated KG1a cells displayed no morphologic changes (data not shown). Similar results were obtained with recombinant human Fas-L used at various doses (0.2-2 μg/mL) (data not shown). These results confirmed that despite high level of Fas expression, KG1a cells were resistant to Fas-induced apoptosis.

Fas clustering in KG1a cells

We addressed whether Fas ligation could induce Fas receptor aggregation in KG1a cells. To resolve this question, we used an immunofluorescence technique coupled to confocal microscopy analysis as reported elsewhere. In these experiments, KG1a cells were or were not stimulated with Fas-L (0.5 μg/mL) for 4 hours, fixed with 4% paraformaldehyde, incubated with ZB4 murine anti-Fas monoclonal antibody or nonimmune mouse IgG1 (data not shown), and stained by FITC-labeled goat–anti-mouse IgG. For this study, ZB4 monoclonal antibody was preferred to CH11 because, unlike CH11, this antibody recognized a Fas epitope distinct from the Fas-L binding site. Jurkat (data not shown) and U937 cells were used as controls. Confocal laser microscopy showed that though untreated KG1a cells exhibited diffuse staining of Fas (Figure 2A), stimulation of cells with Fas-L resulted in Fas aggregation, enabling a dense, patchy staining that was primarily membrane localized (Figure 2C). Similar findings were found in the Fas-sensitive cell lines, as shown in Figure 2B and D, for U937 cells. Because Fas oligomerization appeared to be functional in Fas-activated KG1a cells, we hypothesized that the interruption of Fas signaling was situated immediately downstream of the Fas receptor and that in these cells, for example, some regulators interfered with DISC formation and caspase-8 activation.

![Figure 1. CH11 effect on cell viability.](www.bloodjournal.org)
Caspase-8 activation in KG1a cells

In preliminary experiments, whole-cell lysates were examined by immunoblot analysis and demonstrated that the adapter protein FADD (27-28 kd), procaspase-8 (53-55 kd), and procaspase-3 (32 kd) were present in KG1a cells at a level similar to that of Jurkat cells (Figure 3A). These results showed that the proteins that potentially constitute DISC—ie, Fas, FADD, and caspase-8—are present in KG1a cells.

We next determined whether Fas ligation could lead to the formation of a functional DISC. As shown on Figure 3B, DISC formation was well detected in Fas-L–treated Jurkat cells at 15 minutes. In contrast, incomplete DISC formation was detected in Fas-L–treated KG1a cells because only FADD was observed in the complex. This result suggested that in Fas-L–treated KG1a cells, the defect of DISC formation was caused by the absence of procaspase-8 recruitment. Finally, we examined the generation of procaspase-8 cleavage products in KG1a cells treated with CH11 monoclonal antibody. Therefore, KG1a cells were treated with CH11 (2 μg/mL) for 24 and 48 hours, after which whole-cell lysates were subjected to immunoblotting with a mixture of antibodies directed against procaspase-8 and its p20 and p10 cleavage products. As shown in Figure 3, whereas exposure to CH11 monoclonal antibody resulted in procaspase-8 proteolysis in Fas-sensitive Jurkat cells (Figure 3C), there was no generation of cleavage products in CH11-treated KG1a cells (Figure 3D). The lack of caspase-8 activation may explain why in KG1a cells, Fas ligation was unable to generate caspase-3 cleavage intermediates (Figure 3F). Together these results suggested that in KG1a cells, the lack of Fas-induced apoptosis was related to the presence of negative regulators that interfere with DISC formation and subsequent inhibition of caspase-8 activation. Among different parameters, we speculated that in these cells, PKC activity might play an important role in regulating the formation of functional DISC and Fas-mediated cell death. This was investigated by evaluating the capacity of chelerythrin or calphostin C, 2 known PKC inhibitors, to restore Fas-induced cytotoxicity.

Effect of PKC inhibitors on Fas-mediated cytotoxicity in KG1a cells

KG1a cells were pretreated with either chelerythrin (20 μM) or calphostin C (50 nM) for 1 hour, then were incubated in the presence of CH11 monoclonal antibody for 4 hours. Cell viability was measured by Trypan blue exclusion assay. Under these conditions, neither chelerythrin, calphostin C, nor CH11 monoclonal antibody alone influenced KG1a cell viability (data not shown). As shown in Figure 4A, CH11 monoclonal antibody induced a rapid loss of viability with 50% of residual viable cells at 4 hours in the chelerythrin-pretreated population. In addition, in chelerythrin-pretreated cells, CH11 treatment restored caspase 8 activity (Figure 4B). However, cotreatment with calphostin C and CH11 for 4 hours did not affect KG1a cell viability (Figure 4A) or caspase-8 activity (Figure 4B). Chelerythrin and calphostin C are known to target distinct sites of PKC. Indeed, the former interferes
Fas resistance of KG1a cells. We speculated that this PKC isoform might play an important role in Fas resistance. KG1a cells were pre-incubated with 10 μM antisense (AS) PKCζ or sense (control) oligonucleotides for 48 hours and then were treated or not treated with CH11 (2 μg/mL). (A) PKCζ expression analyzed by Western blot. (B) Fas-induced cell cytotoxicity was evaluated by Trypan blue exclusion assay. Results are expressed as percentage increase in CH11 against IgM-treated cells and are the mean ± SD of 3 independent experiments. *P < .05. (C) Caspase-8 activity was evaluated at 4 hours after CH11 treatment, as described in “Materials and methods.” Results are the mean ± SD of 3 independent experiments. *P < .05. (D, E) Morphology of KG1a cells. (D) Sense oligonucleotide-treated KG1a cells. (E) Antisense oligonucleotide-treated KG1a cells.

Figure 5. Effect of antisense oligonucleotides directed against PKCζ in KG1a cells

PKCζ regulation by Par-4 protein

To further explore the role of PKCζ in Fas-resistance of KG1a cells, we investigated the influence of Par-4 (prostate apoptosis response-4), a known specific regulator of PKCζ.41,42 Western blot analysis revealed that KG1a cells expressed no detectable Par-4 protein, whereas Jurkat cells displayed high Par-4 level (Figure 6A). Hence, KG1a cells were stably transfected by a plasmid containing the full-length Par-4 cDNA sequence. Ten clones were obtained, for which only 2 (clones KG1a/G8 and KG1a/G9) had an immature phenotype (CD34+, CD38−) such as the parental KG1a cell line. Par-4 overexpression in the KG1a/G8 subclone (Figure 6A) resulted in a noticeable reduction of PKCζ activity compared to KG1a cells (Figure 6B), whereas it did not influence PKCζ expression (Figure 6C). Moreover, in KG1a/G8 cells, CH11 induced the activation and the cleavage of caspase-8 and, thus, cytotoxicity and apoptosis (Figure 7). These results suggested that in KG1a cells, low Par-4 expression level and subsequent PKCζ overactivity played an important role in the lack of DISC formation.

Interaction between PKCζ and DISC components

The fact that PKCζ inhibition restored Fas-induced caspase-8 activation in KG1a cells suggested that PKCζ might interact with DISC components. To test this hypothesis, whole-cell extracts were subjected to immunoprecipitation with anti-FADD antibody, and immunoprecipitates were blotted with anti-PKCζ monoclonal antibody. PKCζ was found to interact with FADD (Figure 8A); in parallel, PKCζ immunoextracts prepared from wild-type KG1a cells were able to phosphorylate FADD–agarose complexes (Figure 8B). Furthermore, this phosphorylation is dramatically reduced in Par-4 overexpressed KG1a–G8 cellular extracts (Figure 8B).
Discussion

Our study shows that in KG1a cells, Fas activation results in Fas aggregation, incomplete DISC formation leading to a defect in caspase-8 activation. However, pretreatment with chelerythrin, an inhibitor of all types of PKC isozymes, and, more specifically PKCζ depletion by antisense oligonucleotides or PKCζ inactivation by Par-4 overexpression, restored Fas-induced caspase-8 activity and cytotoxicity. These results suggest that in KG1a cells, PKCζ plays a critical role in altered DISC formation. To the best of our knowledge, the influence of the atypical PKCζ isozyme on Fas signaling has never been reported. However, the role of other PKC isozymes on Fas-induced apoptosis has already been investigated in lymphoid cells. In these studies, it has been shown that Go 6976, a proposed classical PKC isozyme inhibitor, facilitated Fas cytotoxicity in Jurkat cells whereas treatment with phorbol esters significantly reduced Fas-induced caspase-3, PARP cleavage, and apoptosis in T cells.31-33,43-46 Moreover, in the latter studies, it has been described that in the Jurkat T-cell model, phorbol ester-induced PKC stimulation resulted in decreased Fas aggregation whereas in other T-cells, it has been shown that phorbol esters or diacylglycerol reduced Fas expression.44 Because of the specificity of these reagents, which selectively target classical or novel PKC isozymes, it can be assumed that in lymphoid cells, nonatypical PKC isoforms can also efficiently regulate Fas-induced apoptosis. Therefore, it appears that depending on the cellular models, PKC may interfere with Fas signaling through distinct PKC isozymes and mechanisms. However, we observed that, in Jurkat cells, PKCζ overexpression partially inhibited Fas-induced apoptosis (A.d.T., unpublished results, 2001), suggesting that PKCζ may also regulate Fas signaling in nonmyeloid leukemic cells.

The mechanism by which PKCζ inhibited DISC formation in KG1a cells was investigated. Fas, FADD, and caspase-8 expression levels were similar in KG1a/G8 cells, compared to KG1a cells (data not shown). This result suggests that PKCζ does not act by decreasing the expression of DISC protein components. The role of FLIP, a potent negative regulator of DISC formation,10 is also unlikely. Indeed, FLIP expression in KG1a cells was similar to that of Fas-sensitive U937 and Jurkat cells, and Par-4 overexpression had no influence on FLIP levels in KG1a cells (data not shown). These results suggest that FLIP plays a minor role in KG1a cell resistance and that PKCζ does not act on FLIP expression. Based on previous studies that described serine phosphorylation sites on FADD protein,47,48 we hypothesized that PKCζ regulates DISC formation by influencing FADD phosphorylation status. Indeed, our study shows that PKCζ interacts with FADD in vivo and that PKCζ may directly phosphorylate FADD in vitro. Moreover, we found a correlation between FADD phosphorylation status and caspase-8 activation. These results strongly suggest that PKCζ-mediated FADD phosphorylation contributes to caspase-8 inhibition and subsequent Fas resistance in KG1a cells.

Oncogenic Ras and growth factors including platelet-derived...
growth factor or nerve growth factor (TrkA/NGF) may enhance PKCζ activity. 59-61 These signaling pathways are potentially stimulated in AML cells. Therefore, it is possible that these stimuli may reduce Fas-sensitivity of AML cells through a PKCζ-dependent mechanism. It has been documented that PKCζ is a target for phoshoinositide-3 kinase (PI3K) lipid products. 72,73 Interestingly, it has been reported recently that tyrosine kinase receptor-driven PI3K stimulation resulted in the abrogation of FADD-caspase-8 interaction and Fas-induced apoptosis. 54 Whether PKCζ plays a role in PI3K-induced Fas resistance should be investigated.

In this study we also showed that in KG1a cells, the lack of Par-4 expression plays an important role in Fas resistance. Par-4 interacts with the regulatory domain of PKCζ through its leucine zipper domain, and this interaction inhibits the kinase activity. 41 It is generally believed that the negative regulation of PKCζ is the principle mechanism by which Par-4 exerts its pro-apoptotic function though it could also act by modulating Bcl-2 expression and transcription function of WT-1. 55-57 Whereas Par-4 has emerged as a pivotal player in neuronal apoptosis, 42 so far it has received little attention in leukemia. The fact that the monocytic U937 cells, but not KG1a cells, displayed substantial Par-4 expression level suggests that the Par-4 gene is regulated in AML cells. The mechanism by which Par-4 is regulated remains largely unknown. However, it has been reported that oncogenic Ras causes down-regulation of Par-4 in fibroblasts. 58 Therefore, it should be investigated whether Ras, by regulating Par-4 expression, plays an important role in regulating apoptosis induced by Fas, and perhaps by other stress, in AML cells.

To conclude, our study shows that in myeloid leukemic cells, PKCζ and Par-4 are coupled regulators of Fas cell death signaling by interfering with DISC formation. Moreover, because PKCζ is expressed in normal CD34+ cells, 59,60 it should be important to investigate whether this kinase also plays a role in Fas resistance of hematopoietic progenitors.

Acknowledgments
We thank Dr. M. T. Diaz-Meco and Dr. J. Moscat (Madrid, Spain) for the kind gift of Par-4 cDNA, and Dr. C. Bezombes-Cagnac for helpful discussions.

References

10. Tschopp J, Inmier M, Thome M. Inhibition of Fas death signals by FLIPs. Curr Opin Immun. 1998;10:552-556.
Activation of CD95 (APO-1/Fas) signaling by ceramide mediates cancer therapy-induced apoptosis. EMBO J. 1997;16:6200-6208.


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