Absence or Low Expression of Fas-Associated Protein with Death Domain in Acute Myeloid Leukemia Cells Predicts Resistance to Chemotherapy and Poor Outcome

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ABSTRACT

In acute myeloid leukemia (AML), coexpression of death receptors and ligands of the tumor necrosis factor (TNF) receptor/TNF-α superfamily on leukemic cells after chemotherapy is not always accompanied by apoptosis, suggesting that the apoptotic death receptor signaling pathway is disrupted. Because Fas-associated protein with death domain (FADD) is the main adaptor for transmitting the Fas, TNF-related apoptosis-inducing ligand receptors, and TNF receptor 1 death signal, expression of FADD was analyzed by Western blot and immunocytochemistry in leukemic cells of 70 de novo AML patients treated with the European Organization of Research and Treatment of Cancer AML-10 randomized trial before initiation of induction chemotherapy. Thirty seven percent of patients (17 of 46) with FADD negative/low (FADD−/low) leukemic cells had a primary refractory disease compared with 12% of FADD+ patients (3 of 24; P = 0.05), FADD−/low expression was significantly associated with a worse event-free survival [EFS (P = 0.04)] and overall survival (P = 0.04). In multivariate analysis, FADD−/low protein expression was independently associated with a poor EFS and overall survival (P = 0.002 and P = 0.026, respectively). Importantly, FADD−/low protein expression predicted poor EFS even in patients with standard- or good-risk AML (P = 0.009). Thus, we identified low or absent expression of the FADD protein in leukemic cells at diagnosis as a poor independent prognostic factor that can predict worse clinical outcome even for patients with standard- or good-risk AML.

INTRODUCTION

Chemotherapeutic drugs used for leukemia treatment can kill target cells via several mechanisms, including apoptosis. Apoptosis mediated by the Fas and tumor necrosis factor (TNF)-related apoptosis-inducing ligand receptors (TRAIL-Rs) is involved in the antileukemic activity of anthracyclines (1) and etoposide (2), two major cytotoxic drugs for acute myeloid leukemia (AML) therapy. Both were shown to increase TRAIL-R2 [death receptor (DR) 5] expression on human leukemic cell lines and to enhance TRAIL-mediated apoptosis of these cells (3). Moreover, anthracyclines and other drugs up-regulate Fas or Fas ligand (FasL) molecules on tumor cells, which leads to suicide/fragile death of cancer cells (4, 5). Etoposide can activate Fas clustering and consequently Fas-mediated cell death independently of FasL expression (2). However, in most AML patients, leukemic cells in vitro are resistant to Fas-mediated cell death despite expressing the Fas receptor (6), which suggests that the apoptotic Fas signaling pathway is disrupted in AML cells.

Because chemotherapeutic drugs can induce apoptosis of leukemic cells, failure in the apoptotic machinery could lead to chemoresistance and may therefore have an impact on clinical outcome (7). In AML, the expression level of molecules involved in the mitochondria-mediated pathway of apoptosis provides important prognostic information. Although the prognostic value of the expression of bcl-2 antiapoptotic and bax proapoptotic molecules is controversial (8–10), the bax to bcl-2 ratio is of prognostic value in AML patients (11, 12). The prognostic value of the expression of molecules involved in DR-mediated apoptosis pathways has been less studied. However, it has recently been shown that a defect in the caspase activation pathways correlates with resistance of AML patients to chemotherapy (13), suggesting that the apoptotic signaling pathways induced by DRs are disrupted in chemoresistant AML cells.

Engagement of the Fas receptor by three FasL molecules leads to clustering of Fas and consequent recruitment of the Fas-associated protein with death domain (FADD) adaptor protein (14, 15). FADD can in turn activate procaspase 8, which is the initiator of a caspase cascade ultimately resulting in Fas-bearing cell death (16). Although at least two more adaptors for the Fas receptor are known (17, 18), FADD is the main adaptor transmitting the death signal via Fas (19). Moreover, FADD is a key adaptor molecule for numerous DRs because caspase-mediated apoptosis induced by at least TNF receptor 1, DR3, DR4 (TRAIL-R1), and DR5 (TRAIL-R2) needs FADD (20–24). Until now, the role of FADD in drug-induced apoptosis of leukemic cells has been studied mainly in cell lines. In the U937 human leukemia promonocytic cell line, blockage of the DR signaling pathway by transient expression of FADD antisense or by transfection with a vector encoding a FADD dominant negative mutant has been shown to prevent cytotoxic drug-induced apoptosis of these cells (2).

In the present study, we identified the absence or low expression of FADD protein in AML cells at diagnosis as a poor independent prognostic factor. Indeed, complete remission (CR) rate, event-free survival (EFS), and overall survival (OS) were significantly diminished in patients with FADD-negative/low (FADD−/low) leukemic cells (P = 0.05, P = 0.04, and P = 0.04, respectively). Absence or low expression of FADD protein was independently associated with a poor EFS and a poor OS in multivariate analysis (P = 0.002 and P = 0.026, respectively). Importantly, low or absent expression of FADD protein predicted poor EFS even in patients with standard- or good-risk AML (P = 0.009). In summary, we identified low or absent expression of the FADD adaptor molecule as a new independent marker of poor clinical outcome in AML patients.

MATERIALS AND METHODS

Patients. Peripheral blood mononuclear cells [PBMCs (n = 36 patients)] or bone marrow (BM) cells (n = 34 patients) of 70 leukemic patients were collected between September 1988 and September 2002 before the initiation of

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chemotherapy, after patients had given informed consent. This study was approved by the local ethical committee (Comité Consultatif de Protection des Personnes Participant à une Recherche Biomédicale, Paris-Cochin). The mononuclear cells were separated from total blood or BM samples of patients with hyperleukocytosis by Ficoll-Hypaque gradient (Pharmacia Biotech AB, Uppsala, Sweden) and frozen in 10% dimethyl sulfoxide, 90% human AB serum (PAA Laboratories GmbH, Linz, Austria) or used fresh. All of the mononuclear samples contained a majority of tumor cells [minimum, 30% blasts per patient; maximum, 100% blasts per patient; median, 86% blasts per patient (n = 67)] checked by cytological and phenotypical analysis. A cyto- genetic and molecular analysis was performed in all but four patients at diagnosis. Conventional cytogenetic analysis was performed on 24-hour stim- ulated BM cultures with synchronization according to standard procedures. Chromosome analysis was carried on R-banded chromosomes, and karyotypes were described according to the International System for Human Cytogenetic Nomenclature (25). At least 20 mitoses were analyzed before being considered normal. Fluorescent in situ hybridization analyses were performed with commercially available probes (LSI CBFB Dual Color, LSI MLL Dual Color, and LSI BCR/ABL ES Dual Color; Vysis, Downers Grove, IL), according to the manufacturer's instructions. For AFI0, two adjacent AFI0 yeast artificial chromosomes, 807B3 and 815C7 from the Centre d'Etudes du Polyomorphisme Humain library, were labeled with fluorescein isothiocyanate. For CALM, two tetramethyl rhodamine-labeled Centre d'Etudes du Polyomorphisme Humain yeast artificial chromosomes, proximal 785C1 and distal 914D9, had been previously identified to detect CALM rearrangement as a split signal. Dual color analysis was performed as described previously (26). The analysis for AML1-ETO, CBFB-MYH11, and PML-RARα was performed at diagnosis as described previously (27, 28).

**Cell Lines.** The human leukemic myeloid line THP1 was cultured at 37°C in a 5% CO2 atmosphere in RPMI 1640 (Life Technologies, Inc., Cergy Pontoise, France) supplemented with 10% fetal bovine serum, 100 units/mL penicillin (Life Technologies, Inc.), 100 μg/mL streptomycin (Life Technolo- gies, Inc.), and 5 × 10−5 mol/L 2-mercaptoethanol.

**Fluorescence-Activated Cell-Sorting Analysis.** PBMC samples contain- ing a majority of leukemic cells were thawed and incubated at 37°C in a 5% CO2 atmosphere for 1 hour in RPMI 1640 supplemented with 10% human AB serum, penicillin, and streptomycin before use. Fas receptor expression was analyzed by flow cytometry (FACScalibur; Becton Dickinson, San Jose, CA). Sample concentrations were determined using a micro BCA protein assay (Pierce, Rockford, IL), and 30×106/mL serum for 25 minutes at 4°C and then washed in PBS. Phycoerythrin-conju- gated mouse IgG1 antibody [Ab (Immunotech, Beckman Coulter, Inc., Mar- seille, France)] was used as isotype-matched control Ab.

**Western Blotting.** Isolated leukemic cells or cell lines were washed in PBS, and total proteins were extracted with lysis buffer [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 7.8), 1% Nonidet P-40, 1 mmol/L phenylmethylsulfo- nyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL leupeptin], containing a mixture of protease inhibitors (Calbiochem-Novabiochem Corp., La Jolla, CA). Sample concentrations were determined using a micro BCA protein assay reagent kit (Pierce, Rockford, IL), and 30 μg of total proteins were transferred to SDS-PAGE and transferred to polyvinylidene difluoride membrane (New England Nuclear Life Sciences, Boston, MA). Each gel run included a sample of THP1 cells as a positive control. To insure standardization of the positive control, all THP1 cell protein used was from a single, large protein preparation. After blotting, membranes were probed with specific anti-FADD primary Ab [125-140; rabbit IgG (Calbiochem) or rabbit IgG (Cell Signaling Technology, Orange, Saint-Quentin en Yvelines, France)] at 1 μg/mL in Tris-buffered saline (TBS) and 0.1% Tween 20 containing 5% bovine serum albumin overnight at 4°C. The secondary Ab was peroxidase-labeled antirabbit IgG (1:5,000; Amersham Pharmacia Biotech, Orsay, France). To check that an equal amount of proteins from each sample was loaded, the same membrane was probed again with an anti-α-tubulin Ab (clone D1A1; Sigma-Aldrich chemic SARL, Saint Quentin Fallavier, France). Proteins were visualized using the enhanced chemiluminescence technique (Amersham Pharmacia Biotech). Quantification was done by densitometry using Biocat and Bio-Profile BioId software. Leukemic samples were considered to be FADD positive when the numerical value obtained for the FADD signal was greater than or equal to the FADD signal obtained with the THP1 positive control on the same blot. Inversely, leukemic samples were considered to be FADD negative when the numerical value obtained for the FADD signal was less than the FADD signal obtained with the THP1 cells.

**Immunocytochemistry.** Cytopsins were prepared from PBMC (n = 23) or BM aspiration (n = 34) of AML patients at diagnosis. Before staining, cytopsins were slides were fixed for 10 minutes in acetone and incubated for 20 minutes at 0.05 mol/L TBS (pH 7.6) in the presence of 10% normal horse serum and then stained with rabbit polyclonal anti-FADD Ab (125-140) at 10 μg/mL for 45 minutes at room temperature. Then, cytopsins were incubated with biotinylated conjugated antirabbit secondary Ab (Vector Laboratories, Burlingame, CA) followed by phosphatase alkaline-conjugated streptavidin (Amersham Life Science, Arlington Heights, IL). Positive reactivity was revealed by adding Fast-red substrate (Acror Orgonics, Noisy-Le-Grand, France). Cytopsins were counterstained in hemalum (Fisher Scientific, Pitts- burgh, PA) and/or mounted directly in an aqueous mount (Aquateq; Merck, West Point, PA). FADD protein expression was assessed by two independent observers without knowledge of patient outcome or response to treatment. The THP1 cell line was used as positive control.

**Confocal Immunofluorescence Microscopy.** Cytopsins were prepared as described for immunocytochemistry analysis. Before staining, cytopsins were fixed for 30 minutes in PBS with 4% paraformaldehyde and incubated for 20 minutes at 0.05 mol/L TBS (pH 7.6) in the presence of 10% normal horse serum. Cytopsins were then stained for 60 minutes with primary antihuman FADD Ab (125-140), followed by secondary biotin-conjugated antirabbit IgG Ab as described previously. Alexa Fluor 488 conjugate streptavidin (10 μg/mL; Molecular Probes, Inc., Interchim, Montluçon, France) was used to visualize specific staining (30 minutes incubation at room temperature and protected from light). After washings in PBS, sections were mounted with VECTASHIELD medium with 4,6-diamidino-2-phenylindole (Vector Laboratories) to counterstain DNA. Cells were analyzed by confocal fluorescence microscopy (Bio-Rad MRC1024; Bio-Rad, Hercules, CA) equipped with a digital Diapath 300 system. Digital pictures were analyzed using LaserSharp software and processed using Adobe Photoshop. The THP1 cell line was used as positive control.

**Statistical Analysis.** Associations between single variables and remission were assessed by Wilcoxon rank-sum tests for quantitative variables and by the χ2 test or Fisher’s exact test, if more appropriate, for polytomous variables. EFS was measured from diagnosis to disease progression, death from any cause, or date of last follow-up visit. Overall survival was measured from diagnosis to death or date of last follow-up visit. Patient follow-up was updated through January 1, 2004. Survival curves for EFS and OS were plotted by using the Kaplan-Meier method (29) and compared using the log-rank test. All analyses were done with Splus software (Mathsoft, Seattle, WA). All tests were two-sided, with a significance level of 0.05.

The prognostic importance of pretreatment variables, such as age (>40 years and ≤40 years), sex, leukocytosis (>30 × 109 cells/L and ≤30 × 109 cells/L), cytogenetic findings (categorized as high, intermediate, or low risk), and FADD expression (presence and absence), was determined by using the log-rank test. A multivariate Cox regression model was used to determine the relative prognostic importance of several variables (e.g., cytogenetics and FADD expression).

**RESULTS**

**Patients.** Leukemic cells from 70 consecutive patients with de novo AML diagnosed and treated in Hôpital Necker-Enfants Malades or Hôtel-Dieu were included in this study. Patient characteristics are summarized in Table 1. All peripheral blood or BM samples were collected before initiation of induction chemotherapy. Patients were treated with the triple as- sociation of cytarabine (100 mg/m2/d from day 1 through day 10), etoposide (150 mg/m2/d from day 1 through day 5), and an anthra- cycline (50 mg/m2/d daunorubicin, 10 mg/m2/d idarubicin, or 12 mg/m2/d mitoxantrone for 3 days) in accordance with the European Organization of Research and Treatment of Cancer (EORTC) AML-10 randomized trial. The three anthracycline arms gave similar results in terms of outcome in 2,157 patients analyzed (30). Fifty eight patients were randomized and treated in the trial, and 12 were not
randomized but were treated according to the protocol. Patients in first CR as defined in the protocol (31) received a single intensive consolidation course, which consisted of 6 days of intermediate-dose cytarabine and 3 days of the anthracycline, randomly assigned at registration. Patients with a sibling donor were assigned to undergo an autologous BM transplantation in first CR (n = 13). Others received an allogeneic blood or BM transplant. Among the 70 patients, 11 were allografted after they had relapsed. All patients were monitored for 1.2 to 15 years after collection of the cells (median follow-up, 6.5 years). The median EFS and OS for the entire cohort was 9.4 months and 16.8 months, respectively (Fig. 1A; EFS not shown). The course was representative of the natural history of AML patients treated with the EORTC AML-10 protocol in terms of EFS, OS, and prognostic significance of classical risk factors [RFs (Fig. 1B; EFS not shown; ref. 30)].

Fas Protein Is Expressed on Leukemic Cells of Most Acute Myeloid Leukemia Patients. Because chemotherapy could act via a Fas-mediated apoptosis pathway, we looked for Fas expression on AML cells at diagnosis. Flow cytometric analysis of Fas expression showed that Fas was expressed on leukemic cells of eight of nine patients tested (Fig. 2). This result, although obtained on a small number of patients, suggested that failure of Fas-mediated apoptosis induced by chemotherapy would not result from a lack of Fas receptor expression on AML cells. Therefore, we hypothesized that the apoptotic Fas signaling pathway could be disrupted downstream of the Fas receptor in AML cells.

Detection of FADD Protein Expression in Acute Myeloid Leukemia Cells by Immunocytochemistry and Confocal Immunofluorescence Microscopy. In an attempt to statistically validate the previous results, we used a second technical approach allowing the study of a large cohort of patients (n = 57). Thus, FADD expression was analyzed by immunocytochemistry of peripheral blood (n = 23) or BM cytospins (n = 34) collected at diagnosis and correlated with response to chemotherapy. FADD was detected in the THP1 positive cell line (Fig. 3B, top panel), demonstrating that immunostaining is a relevant and simple technique for detecting FADD protein. A representative staining of FADD-positive and FADD-negative AML cells from a chemosensitive patient (patient 21) and a chemoresistant patient (patient 22), respectively, is shown (Fig. 3B, bottom panels).

To confirm these observations, we used a third technique. AML cells of 14 patients were analyzed by confocal immunofluorescence microscopy. FADD was detected in the THP1 positive cell line (Fig. 3C, top left panel). A representative staining of FADD-positive and FADD-negative AML cells from three chemosensitive patients (patients 40, 34, and 24) and a chemoresistant patient (patient 39), respectively, is shown (Fig. 3C, top right and bottom panels).

**Table 1 Patient characteristics**

<table>
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<tr>
<th>Characteristic</th>
<th>Value</th>
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<tr>
<td>Age (y)</td>
<td>41.9 (18.2–59.6)</td>
</tr>
<tr>
<td>WBC count at diagnosis (×10⁹/liter)</td>
<td>22.7 (1.6–600)</td>
</tr>
<tr>
<td>FAB morphology (n)</td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>8</td>
</tr>
<tr>
<td>M1</td>
<td>18</td>
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<tr>
<td>M2</td>
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<td>M4</td>
<td>16</td>
</tr>
<tr>
<td>M5</td>
<td>13</td>
</tr>
<tr>
<td>M6</td>
<td>2</td>
</tr>
<tr>
<td>Cytogenetic analysis [n (% of total)]</td>
<td></td>
</tr>
<tr>
<td>Good risk (RF1)</td>
<td>11 (16)</td>
</tr>
<tr>
<td>Inv(16)</td>
<td>4</td>
</tr>
<tr>
<td>t(8;21)</td>
<td>7</td>
</tr>
<tr>
<td>Standard risk (RF2)</td>
<td>42 (60)</td>
</tr>
<tr>
<td>No abnormality</td>
<td>39</td>
</tr>
<tr>
<td>Other abnormalities</td>
<td>3</td>
</tr>
<tr>
<td>Poor risk (RF3)</td>
<td>13 (18)</td>
</tr>
<tr>
<td>Complex, 5–7</td>
<td>4</td>
</tr>
<tr>
<td>t(1q23)</td>
<td>6</td>
</tr>
<tr>
<td>t(6;9)</td>
<td>1</td>
</tr>
<tr>
<td>t(10;11)</td>
<td>2</td>
</tr>
<tr>
<td>Failure</td>
<td>4 (6)</td>
</tr>
</tbody>
</table>

NOTE: Age, N = 70 patients. WBC count, n = 68 patients. FAB morphology based on ref. 55. Abbreviations: WBC, white blood cell; FAB, French-American-British; RF, risk factor.

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Fig. 1. Survival of AML patients treated with chemotherapy. A, OS of the entire cohort. B, OS of good-risk (RF1), standard-risk (RF2), and poor-risk (RF3) patients. Median OS of RF1, RF2, and RF3 patients was not reached, 18.1, and 11.1 months, respectively (median followup, 6.5 years). Median EFS of RF1, RF2, and RF3 patients was not reached, 9.1, and 6.7 months, respectively (data not shown). Tick marks indicate patients who were alive at last follow-up.
FADD EXPRESSION AS A NEW PROGNOSTIC FACTOR IN AML

Immunostaining (immunocytochemistry or confocal immunofluorescence microscopy) and Western blot analysis gave concordant results for the 13 patients studied (Fig. 3D), suggesting that both techniques can be used to detect FADD protein expression in AML cells.

Absent or Low FADD Protein Expression in Leukemic Cells at Diagnosis Is a Major Independent Poor Prognostic Factor for Acute Myeloid Leukemia Patients. Results obtained by Western blotting (n = 31) and/or immunocytochemistry (n = 57) are summarized in Table 2.

Classical risk factors are described for FADD + and FADD -/low populations: the median age and white blood cell count at diagnosis were 35.5 versus 44.5 years and 40.7 × 10⁹ versus 16.2 × 10⁹ cells/liter in FADD + and FADD -/low patient groups, respectively. Interestingly, leukocytosis, which is a classical poor-risk factor in AML, was significantly higher for FADD + patients (P = 0.02, Wilcoxon rank-sum test; Table 2). On the other hand, the number of patients with good-, standard-, and poor-risk factors was not significantly different between FADD + and FADD -/low populations (good-risk [RF1]/standard-risk [RF2]/poor-risk [RF3] populations: 3/15/6 versus 8/27/7, respectively). There was a higher number of patients receiving allogeneic BM transplantation in the FADD + group, translating the fact that fewer patients were in CR and thus eligible for allogeneic BM transplantation in the FADD -/low group (Table 2).

In univariate analysis, primary refractory disease was associated with absence or low expression of FADD protein in AML cells at diagnosis. Indeed, 17 of 46 (37%) patients with FADD -/low leukemic cells versus 3 of 24 (12%) patients with FADD + AML cells did not reach CR (P = 0.05; Table 2). These observations had a major impact on EFS and OS (Fig. 4A and B, respectively) because the median EFS was 42 months for FADD + patients versus 7.7 months for FADD -/low patients (P = 0.04; Fig. 4A), and the median OS was 45.1 months for FADD + patients versus 14.3 months for FADD -/low patients (P = 0.04; Fig. 4B). Thus, absent or low expression of FADD protein in leukemic cells at diagnosis indicates a poor prognosis with regard to the achievement of CR, EFS, and OS.

In multivariate analysis using a Cox regression model, the only independent variables associated with a poor EFS and poor OS were poor-risk cytogenetics (RF3; P = 0.005 and P = 0.004, respectively) and the absent or low FADD protein expression (P = 0.002 and P = 0.026, respectively). Thus, absent or low FADD protein expression in leukemic cells at diagnosis was an independent prognostic factor in this cohort of AML patients.

Absent or Low FADD Protein Expression Is a New Potent Prognostic Factor for Evaluating Standard- or Good-Risk Acute Myeloid Leukemia. To analyze whether absent or low FADD protein expression at diagnosis can be predictive of clinical outcome of patients with standard- or good-risk AML, we focused on RF1 (good-risk population, n = 11) and RF2 (standard-risk population, n = 42) populations. We also included in this study the four patients for whom cytogenetic analysis could not be successfully performed. Among the 57 patients studied, 15 of 39 (38%) with FADD -/low leukemic cells versus 2 of 18 (11%) with FADD + AML cells have a primary refractory disease (P = 0.015, χ² test). Moreover, low/absent FADD protein expression predicted poor EFS in patients with standard- or good-risk AML and a trend for a poor OS. The median EFS was 45.1 months for FADD -/low patients versus 8.1 months for FADD + patients (P = 0.009; Fig. 4C), and the median OS was 45.1 months for FADD -/low patients versus 16.8 months for FADD + patients (P = 0.08; Fig. 4D), indicating that absent or low FADD protein expression can identify a subgroup of patients with poor prognosis among patients that have standard- or good-risk AML.

DISCUSSION

In the present study, we tried to identify mechanisms responsible for resistance of AML cells to DR-mediated apoptosis induced by induction chemotherapy. Because FADD is a key adaptor molecule for the Fas, TRAIL, and TNF-α receptors transmitting the apoptotic signal, we looked at FADD protein expression in leukemic cells of patients at diagnosis and correlated the results with the response to treatment in terms of CR rate, EFS, and OS. This retrospective study concerned a population of 70 consecutive patients with de novo AML treated with the EORTC AML-10 randomized trial. All had typical features of adult AML and are representative of the disease in terms of EFS, OS, and prognostic significance of classical RFs. Here we identified absent or low FADD protein expression in leukemic cells at diagnosis as a new independent prognostic factor for poor response of AML cells to chemotherapy. In our cohort of patients, the absence of FADD protein expression was not due to an absence of FADD mRNA, as assessed by reverse transcription-polymerase chain reaction (data not shown). Thus, it is probable that this new marker will not be identified by techniques targeting gene expression, including complementary DNA microarrays (32, 33). We have shown previously that loss of FADD protein, but not mRNA, occurs in adenomatous/adenocarcinomatous mouse thyroid gland or during in vitro culture of thyroid cells (34). In this last case, we have demonstrated that lack of FADD protein expression did not result from degradation by the proteasome pathway (34). We are currently investigating the exact mechanism accounting for the absence of FADD protein expression in AML cells from chemoresistant patients.

Quantification of various molecules involved in apoptotic pathways has been studied to predict response of leukemia patients to chemotherapy (7). For instance, low Fas protein expression on blast cells has been associated with high resistance of AML patients to chemotherapy (35). Consistent with this study, the ability of anthracyclines to
Fig. 3. Absence or low FADD protein expression in AML cells is correlated with response to chemotherapy. A. Western blot analysis of FADD protein expression in leukemic cells of AML patients at diagnosis. The THP1 cell line was used as a positive control. Protein size is indicated. Bands obtained using Western blot were quantified by densitometry (OD, absorbance), and results are expressed in AU. B and C, detection of FADD protein expression in AML cells at diagnosis by (B) immunocytochemistry and (C) confocal immunofluorescence microscopy. The THP1 cell line was used as a positive control. Positive reactivity was assessed by comparing staining of cells obtained with anti-FADD Ab with the background staining obtained with biotinylated conjugated secondary Ab alone (control Ab). No or low staining or staining of only a few leukemic cells (<5%) was considered negative. Strong staining or intermediate staining of the majority of cells (>95%) was considered positive. For immunocytochemistry analysis, THP1 and FADD-negative AML cytospins were counterstained in hemalun (blue), whereas FADD-positive AML cytospins were either counterstained in hemalun (data not shown) or observed directly to ensure a better visibility. Positive staining appeared in pink. For confocal immunofluorescence microscopy, cytospins were counterstained with 4′,6-diamidino-2-phenylindole (blue), and positive staining appeared in green. D, comparison of FADD protein expression detected in AML cells of 13 patients at diagnosis by immunostaining [immunocytochemistry (ICC) and/or confocal fluorescence microscopy (CFM)] and Western blot (WB) analysis. † results provided from three different blots were normalized to obtain an absorbance value of 50,000 AU for the THP1 positive control. Western blot analysis of α-tubulin was used as a control to check the amount of proteins from each sample loaded on SDS-PAGE. † indicates that leukemic cells expressed a low level of FADD protein compared with THP1 cells. ND, not determined.
enhance Fas expression and Fas-mediated apoptosis of blast cells in vitro was correlated with high CR rates of patients with acute leukemia after chemotherapy (36). In our study, we found that Fas protein was expressed on leukemic cells of eight of nine patients at diagnosis, suggesting that in our cohort of patients, Fas expression does not seem to be the best marker to predict response of AML patients to chemotherapy. Thus, the prognostic value of Fas receptor expression on leukemic cells may depend on different variables, including type (37) and state of development of the disease.

The role of caspases, and particularly caspase 3, in chemotherapeutic drug-induced apoptosis has been established (38, 39). As a consequence, the integrity of the caspase pathway has been studied in leukemic cells and correlated with the clinical outcome of patients, but results are still controversial (40, 41). However, it has recently been shown that a functional defect in the caspase activation pathway was a prognostic factor for resistance of AML cells to chemotherapy (13). Because FADD is necessary for procaspase 8 activation and downstream activation of effector caspases such as caspase 3, these later data are consistent with our finding (20). Furthermore, our data emphasize the fact that the extrinsic apoptosis pathway may act synergistically with the intrinsic apoptosis pathway involving caspase 9 in chemotherapy-induced cell death of AML cells (7).

Absence of FADD protein could have numerous effects on AML cells that predominantly result in a growth/survival advantage. First, because FADD is a common signaling molecule for numerous death pathways, absence of FADD protein expression confers multiple

### Table 2 Characteristics of patients with FADD<sup>+</sup> and FADD<sup>-</sup> AML cells

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>Patients with FADD&lt;sup&gt;+&lt;/sup&gt; leukemic cells</th>
<th>Patients with FADD&lt;sup&gt;-&lt;/sup&gt; leukemic cells</th>
<th>p-value</th>
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<tr>
<td>Age (y) Median (range)</td>
<td>41.9</td>
<td>35.5 (18.5–58)</td>
<td>44.5 (18.2–59.6)</td>
<td>0.086*</td>
</tr>
<tr>
<td>WBC count at diagnosis (×10&lt;sup&gt;9&lt;/sup&gt;/liter) Median (range)</td>
<td>22.7 (n = 68)</td>
<td>40.7 (2.5–241) (n = 24)</td>
<td>16.2 (1.6–600) (n = 44)</td>
<td>0.02*</td>
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<tr>
<td>Risk factor (RF1/RF2/RF3)</td>
<td>11/42/15</td>
<td>3/15/6</td>
<td>8/2/7</td>
<td>0.34†</td>
</tr>
<tr>
<td>Allografted [n (% of total)]</td>
<td>24 (34)</td>
<td>12 (50)</td>
<td>12 (26)</td>
<td>0.045†</td>
</tr>
<tr>
<td>Primary refractory disease [n (% of total)]</td>
<td>20 (29)</td>
<td>3 (12)</td>
<td>17 (37)</td>
<td>0.05†</td>
</tr>
</tbody>
</table>

Abbreviation: WBC, white blood cell.
* Wilcoxon’s rank-sum test.
† χ² test.

Fig. 4. Prognostic impact of absent or low FADD protein expression in leukemic cells at diagnosis on clinical outcome of AML patients and standard- and good-risk AML patients.

Absence of FADD protein could have numerous effects on AML cells that predominantly result in a growth/survival advantage. First, because FADD is a common signaling molecule for numerous death pathways, absence of FADD protein expression confers multiple
resistance to DRs. Apoptosis induced by at least Fas, TNF receptor 1, DR3, DR4, or DR5 needs the FADD adaptor to transduce the death signal (20–24). As a consequence, all these apoptotic pathways may be blocked in AML cells. Interestingly, Fas-, TNF receptor 1-, and TRAIL-R– mediated cell death resistance has been reported in AML cells and human leukemia cell lines despite normal receptors expression (6, 42–45). Second, expression of the FasL molecule can contribute to leukemic immune escape (46). Thus, FADD could have a key role in regulating tumor progression (47). Third, although the major role of the Fas pathway is to induce apoptosis on engagement of FasL with the Fas receptor, it has also been demonstrated that Fas signaling can result in proliferation, depending on the type of cell and the environmental conditions (48–50). In tumors of hematologic and nonhematologic origin, treatment with anti-Fas Ab can promote cell growth instead of apoptosis (51). We have shown previously that FADD protein expression is lost during the development of thyroid adenoma and adenocarcinoma and that agonistic anti-Fas Ab accelerates Fas+ FADD+ thyroid follicular cell growth in vitro (34). Thus, Fas and probably other DRs signaling in the absence of FADD could lead to proliferation of AML cells, contributing to disease progression. This hypothesis is consistent with the high secretion of TNF-α by leukemic cells of some AML patients of our cohort (data not shown).

Many treatments have been used in attempts to render cancer cells sensitive to DRs and particularly Fas-mediated apoptosis (52, 53). In this study, most AML patients were treated in accordance with the EORTC AML-10 randomized trial consisting of triple association of cytarabine, etoposide, and an anthracycline. Both etoposide and anthracyclines can induce, among other consequences, Fas signaling pathway that lead to suicide/fatricide death of Fas+ AML cells (4, 5), and sensitization to TRAIL-R2–mediated cytotoxicity (3). However, one could expect that in the absence of FADD, Fas and TRAIL signaling is not always deleterious for cancer cells, underlining the importance of characterizing FADD protein expression status in AML cells at diagnosis. Therefore, we showed here for the first time that FADD protein was absent in leukemic cells at diagnosis of most AML patients, potentially contributing to chemotherapy failure. In addition to the major prognostic value of FADD protein, the absence of FADD protein could have a physiopathological role in AML progression and represent a new therapeutic target. Indeed, it has recently been shown that human tongue carcinoma cell lines express a very low level of FADD-mediated activation of the Fas death pathway by anticancer drugs. J Biol Chem 1999;274:7987–92.


47. Newton K, Harris AW, Strasser A. FADD/MORT1 regulates the pre-TCR checkpoint and can function as a tumour suppressor. EMBO J 2000;19:931–41.
Absence or Low Expression of Fas-Associated Protein with Death Domain in Acute Myeloid Leukemia Cells Predicts Resistance to Chemotherapy and Poor Outcome

Léa Tourneur, Stéphanie Delluc, Vincent Lévy, et al.


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