Functional Blocks in Caspase Activation Pathways Are Common in Leukemia and Predict Patient Response to Induction Chemotherapy

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ABSTRACT

Defects in apoptosis mechanisms contribute to chemoresistance in malignancy. However, correlations of apoptosis-regulating proteins with clinical outcome in cancer patients are variable, presumably reflecting the difficulty of using static tests of gene expression in a scenario influenced by a dynamic interplay of multiple pro- and antiapoptotic molecules. Therefore, we assessed the functional integrity of apoptosis pathways in intact primary leukemia cells and correlated the functional status of these pathways with clinical outcome. Active apoptogenic proteins were introduced into primary leukemia cells by electroporation followed by measurement of active caspases by flow cytometric techniques. Cytochrome c was introduced to activate the intrinsic (mitochondrial) pathway, whereas caspase-8 was introduced to activate the extrinsic (death receptor) pathway. In a series of 24 patients with acute myeloid leukemia, 79% had a block in at least one pathway, indicating that defects in caspase activation mechanisms are common in patients with leukemia. Simultaneous blocks in both pathways correlated with chemoresistant disease (92% of patients with chemoresistant disease versus 33% of patients with chemosensitive disease, P = 0.005) and decreased overall patient survival (35% versus 89% 1-year survival; P = 0.02). Simultaneous blockage of the intrinsic and extrinsic pathways could be explained by a defect located at a point of convergence of the two pathways, probably related to overexpression of endogenous inhibitors of the effectors-caspases, rather than decreased levels of these proteases. This study supports the importance of apoptosis pathways in determining response to chemotherapy and suggests that functional defects in caspase activation are prognostic in patients with leukemia.

INTRODUCTION

Early identification of patients with chemoresistant AML who are destined to fail to achieve remission after induction chemotherapy is important, so that they may be offered more aggressive or alternate therapy at disease onset. Currently, one of the best predictors of response to therapy in AML is cytogenetic abnormalities, but 70% of patients have intermediate risk cytogenetics, thus limiting the clinical utility of cytogenetics (1). Therefore, a need exists for identification of alternative molecular predictors of response to therapy.

Apoptosis (programmed cell death) mechanisms are hypothesized to play an important role in tumor cell responses with cancer-associated defects in apoptosis mechanisms contributing to chemoresistance (reviewed in Refs. 2, 3). However, at present, the prognostic utility of measurements of pro- and antiapoptotic molecules for predicting clinical outcome and response to chemotherapy is uncertain. For example, in patients with leukemia, most studies indicate that increased levels of the antiapoptotic protein Bcl-2 correlate with chemoresistant disease and decreased overall survival (4, 5). However, in some studies, increased Bcl-2 has no impact (6, 7) on clinical outcome or is even associated with improved survival (8). These discrepancies may indicate that response to chemotherapy is primarily dependent on factors other than the classical apoptotic family members. For example, response to therapy may be dictated primarily by the ability of chemotherapeutic agents to induce irreparable DNA damage (9, 10) or disrupt the cell cycle (11, 12) rather than activate apoptosis pathways. Alternatively, discrepancies in studies of individual apoptotic proteins may reflect the difficulty of using single variables such as Bcl-2 to predict patient outcome. In this regard, response to therapy is likely dictated by the interplay of a variety of pro- and antiapoptotic molecules that determine the functional integrity of apoptosis pathways. Thus, it would be desirable to assess the functional integrity of apoptosis pathways in primary leukemia cells and determine whether competency of apoptosis pathways is predictive of clinical outcome.

Using electroporation to introduce apoptogenic proteins into cells, we have assessed the functional status of two major pathways for caspase activation in acute leukemia cells. We observed that functional blocks in caspase pathways are common in patients with leukemia. Furthermore, simultaneous blocks in both the intrinsic (mitochondria) and extrinsic (death receptor) pathways predict for chemoresistant disease, and decreased patient survival. The findings suggest that a function-based approach to assessing the integrity of apoptosis pathways can provide insight into biological and clinical differences in leukemia response to chemotherapy.

MATERIALS AND METHODS

Patient Samples. Peripheral blood samples were obtained from patients with AML who consented to donation of a research sample. Mononuclear cells were separated by density centrifugation and were stored in liquid nitrogen in the leukemia tumor bank at Princess Margaret Hospital (Toronto, Ontario, Canada). We restricted our samples to those drawn before the initiation of induction chemotherapy and those with a blast count >80%. From the tumor bank, we selected 12 consecutive samples from patients with chemosensitive disease and 12 consecutive samples from patients with chemoresistant disease that met our inclusion criteria. Patients with chemosensitive disease achieved complete remission after induction chemotherapy, whereas patients with chemoresistant disease did not and died from AML. Patients who died during induction chemotherapy before determination of chemosensitivity were not included. All of the patients received cytarabine, 100 mg/m² for 7 days, and daunorubicin, 45–60 mg/m² for 3 days, as initial induction chemotherapy. Patients with chemoresistant disease who failed to respond to induction chemotherapy proceeded to receive either palliative care or an additional unsuccessful attempt at induction, but did not receive an allogeneic blood or marrow transplant. Cells were thawed 36 h before treatment, and cultured in Iscove’s modified Dulbecco’s medium supplemented with 20% fetal bovine serum, penicillin, and streptomycin. Cells were grown at 37°C with 5% CO₂ in a humidified atmosphere. Approval for the study was obtained from the local ethics review boards.

Assessment of the Caspase Activation Pathways in Intact Leukemia Cells. To assess the functional status of caspase activation pathways in leukemic cells, each pathway was activated individually followed by measure-
ment of effector caspase activity as described previously (13). Using recombinant caspase-8 (produced in bacteria and purified as described in Ref. 14) to activate the extrinsic tumor necrosis factor-related pathway and cytochrome c (Sigma, Inc., Milwaukee, WI) to stimulate the intrinsic pathway, caspase-activating proteins were introduced into the primary AML cells, Jurkat, or K562 cells by electroporation after empirically optimizing conditions. The amounts of caspase-8 and cytochrome c used for these assays were empirically determined in pilot experiments using at least two sensitive AML cell specimens. The amounts of activator used represent the minimal concentration of protein necessary to reach maximal caspase activation in this assay.

Cytocrome c (60 μM) or caspase-8 (12 nM) was added to leukemia cells (2.5 × 10^9) in 0.5 ml of RPMI 1640 without serum or antibiotics, along with marker proteins PE-BSA (4 μg/ml) or PE-streptavidin (4 μg/ml; Molecular Probes, Eugene, OR) for identifying successfully transduced cells. The cell-protein mixtures were then transferred to electroporation cuvettes (Bio-Rad, Hercules, CA; 0.4-cm diameter), kept on ice for 10 min, and subjected to electroporation (Gene Pulser II; Bio-Rad) using 625 V/cm and 900 μF capacitance for AML cells, and 500 V/cm and 900 μF for Jurkat and K562 cells. Cells were maintained on ice for 30 min after electroporation, transferred to tubes, and cultured at 37°C in 5% CO₂ in humid conditions for 1 h as caspase activation was assessed. Normalization of protein concentration by addition of unlabeled BSA was determined to have no effect on caspase activation.

**Caspase Activation Assay.** Caspase-3-like effector caspase activity was identified with CaspaTag (Intergen, New York, NY) according to the manufacturer’s instructions. CaspaTag is a cell permeable FITC-labeled peptide that selectively and irreversibly binds active caspase-3. The CaspaTag assay is based on the well-described observation that caspase activation occurs before loss of outer membrane integrity and positive staining with viability dyes such as 7-AAD. After incubation with CaspaTag for 1 h, cells were washed, resuspended in Iscove’s modified Dulbecco’s medium with 20% fetal bovine serum, and analyzed by flow cytometry. For the purpose of dichotomizing the data, a caspase activation pathway was deemed intact if the introduction of the caspase-activating protein increased the percentage of cells containing active effector caspases at least 10% above the baseline compared with cells electroporated with control proteins. By selecting a cutoff of 10%, all of the positive samples had active effector caspase levels at least 2-fold higher than the BSA control.

**Flow Cytometry.** Cells were stained with the viability dye 7-AAD (final concentration 2 μg/mL; Sigma Inc.) for 5 min at room temperature just before flow cytometry analysis (FACScan Immunocytometry system; Becton Dickinson, San Jose, CA). FITC (Channel 1), PE (Channel 2), and 7-AAD (Channel 3) fluorescence were measured, and the Cell Quest program was used to analyze these data. PE-positive and 7-AAD-negative cells were gated, and the percentage of gated cells expressing FITC was determined. Cells to which the PE-markers were added but not electroporated were used to determine the positive and negative cutoffs for the different fluorescent labels. These cells were treated with CaspaTag as described and stained with 7-AAD just before flow cytometry. Detection levels for the three fluorescence channels were based on cells treated with PE-BSA, CaspaTag, and 7-AAD but not electroporated. To achieve uniformity among samples, positive and negative cutoffs for CaspaTag fluorescence were based on unelectroporated controls, and were set so that 5–10% of cells electroporated with BSA alone were positive.

**Assessment of Caspase Activation in Cytotoxic Extracts.** Cytotoxic extracts were prepared from primary AML cells and leukemia cell lines as described previously (15, 16). Briefly, cells were washed in PBS and then resuspended in an equal volume of hypotonic lysis buffer (20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, and 1 mM DTT). Cells were incubated on ice for 20 min and then disrupted by 15 passages through a 30-gauge needle. Cell extracts were clarified by centrifugation at 16,000 × g for 30 min. To initiate caspase activation, 10 μM horse heart cytochrome c (Sigma) with 1 mM dATP or purified recombinant caspase-8 (10 μM) was added to cell extracts (10 μg of total protein), and mixtures were incubated at 30°C for 30 min. Hydrolysis of fluorescent peptide, Ac-DEVD-AFC, was measured as an indicator of caspase activation, as described previously (15, 16). Activated extracts were added to Ac-DEVD-AFC in caspase buffer (50 mM HEPES (pH 7.4), 10% sucrose, 1 mM EDTA, 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid, 100 mM NaCl, and 10 mM DTT). Hydrolysis was measured over 20 min by release of AFC (excita-

tion = 405 nm, emission = 510 nm) using a spectrofluorometer (IFMax; Molecular Devices, Sunnyvale, CA) in the kinetic mode.

**Immunoblot Assays.** Protein extracts were prepared from primary AML and leukemia cell lines. Cells were washed with PBS and resuspended in lysis buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, and 5 mM EDTA) containing protease inhibitors (Complete tablets; Roche, Indianapolis, IN). Protein concentrations were determined by the Bradford assay (17). Immunoblot assays were performed as described previously (18). Briefly, equal amounts of protein were subjected to SDS-PAGE (4–20% gradient gels from ISC BioExpress, Kaysville, UT), followed by transfer to nitrocellulose membranes. Membranes were probed with polyclonal rabbit-antihuman caspase 3 (1:1000 v/v; Ref. 19), monoclonal mouse-antihuman XIAP (0.25 μg/ml; Transduction Laboratories, Lexington, KY), monoclonal rabbit anti-Apaf-1 (0.5 μg/ml; Cayman Chemical, Ann Arbor, MI), monoclonal mouse anti-FLIP (1.5 V/v of hybridoma supernatant; a gift from Marcus Peters, University of Chicago, Chicago, IL), mouse monoclonal anti-b2-actin (1:2000 v/v; Sigma Inc.), and monoclonal mouse anti-b-actin (1:3000 v/v; Sigma Inc.). Secondary antibodies consisted of horseradish peroxidase-conjugated goat antirabbit IgG or goat antimouse IgG (Bio-Rad). Detection was performed by the enhanced chemiluminescence method (Amersham, Arlington Heights, IL). The intensity of the autoradiograph bands was quantified by densitometry (Alpha Innotech, San Leandro, CA).

**Statistical Analysis.** Differences between groups of patients with chemosensitive or chemoresistant disease, and between patients with blocked or intact caspase pathways were determined by χ² analysis and unpaired t test. A dichotomous multiple regression analysis was used to determine factors that predicted response to treatment. Kaplan-Meier analysis (20) and a univariate Cox proportional hazards model estimated survival.

**RESULTS**

**Electroporation Can Efficiently Transduce Proteins into Primary Leukemia Cells.** We used electroporation to transduce proteins into primary leukemia cells. To demonstrate protein transduction with this technique, AML cells were electroporated with 2 μg/ml FITC-conjugated BSA using an electrical field of 625 V/cm and a capacitance of 900 μF. The cells were then washed, stained with 7-AAD, and analyzed by flow cytometry. Using 7-AAD dye exclusion to gate on viable cells that exclude this dye, FITC uptake among the gated cells was measured. In a series of AML samples, (n = 11) mean uptake of FITC-BSA among the viable AML cells was 65 ± 22% with a mean loss of viability above the unelectroporated control of 36 ± 22%. Analysis of electroporated cells by confocal microscopy confirmed that the FITC-BSA protein was located inside the cells and not merely adsorbed to the cell surface. Also, no FITC-BSA uptake was observed in control unelectroporated cells (data not shown). To determine the effects of molecular mass on transduction efficiency, we electroporated AML cells with FITC-Dextran (10,000 kDa), FITC-BSA (66,000 kDa), or FITC-goat antimouse IgG (150,000 kDa). Transduction efficiencies differed by ≤13% among these various molecules, indicating that similar efficiencies of uptake could be achieved for molecules of many masses.

**Assessment of the Functional Integrity of Caspase Activation Pathways in Intact Cells.** Two major pathways for activating caspase-family cell death proteases have been described. The extrinsic pathway is triggered by tumor necrosis factor family receptors and results in activation of the upstream initiator protease, caspase-8. The mitochondrial/intrinsic pathway involves release of cytochrome c into the cytosol where it binds caspase-activator Apaf-1, triggering activation of initiator protease, caspase-9 (reviewed in Ref. 21). These two pathways converge on downstream effector caspases, which then induce apoptosis. We introduced the apoptogenic proteins cytochrome c or active caspase-8 into primary leukemia cells. Activation of downstream effector caspases was then measured using a cell-permeable fluorescent peptide that selectively and irreversibly binds effec-

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tor caspases such as caspase-3 and -7 (CaspaTag). PE-labeled BSA was included during electroporation in these experiments to identify cells that had been successfully electroporated. After electroporation, cells were stained with 7-AAD and then analyzed by flow cytometry. The percentages of leukemia cells containing the CaspaTag substrate above background levels were enumerated, after gating on viable (7-AAD negative), PE-positive cells.

In a representative AML specimen, (patient sample 1), introduction of caspase-8 and cytochrome c activated effector caspases in $27\% \pm 5\%$ and $31\% \pm 5\%$ of cells, respectively, whereas introduction of the BSA control activated effector caspases in only $7\% \pm 1\%$ of cells. We empirically determined that this specimen had intact extrinsic and intrinsic pathways (Fig. 1A). In contrast, in some patient samples (e.g., sample 2), caspase activity did not increase above the BSA control after introduction of either caspase-8 or cytochrome c, and we empirically determined that this specimen had blocked extrinsic and intrinsic pathways.

To corroborate the functional status of the caspase-activation pathway in intact AML cells, we measured caspase activation in cytosolic extracts. Cytochrome c or caspase-8 was added to cytosolic extracts prepared from patient samples 1 and 2, followed by measurement of effector caspase-induced cleavage of Ac-DEVD-AFC (Fig. 1B). In patient sample 1 that we deemed to have intact activation pathways, we observed significant increases in caspase-activation after addition of cytochrome c or caspase-8 to cytosolic extracts. In contrast, only minimal increases in caspase activation were observed after the addition of the activators to cytosolic extracts from patient sample 2 that we deemed to have blocked activation pathways. Therefore, the intracellular assessment of the status of the caspase activation pathways correlates with measurements in cytosolic extracts.

To assess the functional status of caspase activation in normal hematopoietic cells, cytochrome c and caspase-8 were introduced into magnetically separated normal CD34+ cells. In a representative experiment, introduction of cytochrome c and caspase-8 increased effector caspase activity $31\%$ and $34\%$ above the BSA control, respectively (Fig. 1C). Thus, we deemed that normal CD34+ cells have both caspase activation pathways intact.

The specificity of the caspase activation was confirmed by cotrans-

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**Fig. 1.** Stimulation of caspase activation pathways in primary AML cells. The intrinsic and extrinsic caspase activation pathways were activated individually followed by the measurement of effector caspase activity. Cytochrome c (60 μM) or caspase-8 (12 nM) were cotransduced into primary leukemia cells (2 × 10^5) with PE-BSA (4 μg/ml; to identify cells that had been transduced successfully). Cells were stained with the viability dye 7-AAD and analyzed by flow cytometry. Viable (7-AAD-negative), PE-positive cells were gated, and effector caspase activity was measured among gated cells with CaspaTag, a FITC-labeled cell permeable peptide that irreversibly binds active caspase-3. A, representative dot-plots (Y axis = log-fluorescence; X axis = forward scatter) of effector caspase-3 activity in primary AML samples (patient sample 1 = pt. 1, and patient sample 2 = pt. 2) are presented. The horizontal line represents the cutoff for CaspaTag-positive cells. B, the functional integrity of the caspase activation pathways was determined in cytosolic extracts (10 μg protein). Either caspase-8 or the combination of cytochrome c and dATP was added to cytosolic extracts from patient samples 1 and 2. The extracts were incubated at 30°C for 30 min, followed by measurement of DEVD cleavage over time. C, a representative dot-plot (Y axis = log fluorescence; X axis = forward scatter) of effector caspase-3 activity in primary AML samples (patient sample 1 = pt. 1, and patient sample 2 = pt. 2) are presented. The horizontal line represents the cutoff for CaspaTag-positive cells. D, the specificity of caspase activation was confirmed by cotransducing the BIR3-RING of XIAP (20 nM), Crm A (2 μM), or full-length XIAP (0.5 μM), along with specific caspase activators into primary leukemic cells or by pretreating the cells with zVAD-fmk (100 μM), followed by electroporation of the caspase activators. A representative bar graph of the increase in caspase activation above the BSA-control is shown. Cyr-c, cytochrome c; Casp-8, caspase-8.
duding either CrmA, a viral protein that binds and inhibits of caspase-8 (14), or BIR3-RING, a fragment of the human XIAP protein that specifically inhibits caspase-9 (22, 23). Coelectroporation of CrmA (2 μM) or BIR3-RING (20 nM) inhibited caspase activation induced by caspase-8 and cytochrome c, respectively (Fig. 1D). In contrast, CrmA failed to inhibit cytochrome c-induced caspase activation, and BIR3-RING did not suppress caspase-8 induced effector caspase activation. Likewise, coelectroporation of full-length XIAP (0.5 μM) or pretreatment with the broad-spectrum caspase-inhibitor zVAD-fmk (100 μM final concentration) inhibited both cytochrome c and caspase-8-induced effector caspase activation (Fig. 1D).

To validate fluorescence-activated cell sorter-based assessment of the caspase-activation pathways in intact primary AML cells, we extended the results to Jurkat and K562 leukemia cell lines, as they represent leukemia cell lines with likely intact and blocked caspase activation pathways, respectively (24–26). K562 cells had molecular defects in both caspase activation pathways, with lower levels of Apat-1, higher levels of XIAP, and higher levels of the caspase-8 inhibitor FLIP compared with Jurkat cells. Consistent with these findings, Jurkat cells demonstrated more apoptosis and cell death compared with K562 cells after treatment with etoposide (activator of the intrinsic pathway) or anti-Fas antibody (activator of the extrinsic pathway; data not shown). Caspase activation pathways were activated in these intact cells and in cytosolic lysates as described previously. In a representative experiment, introduction of cytochrome c and caspase-8 into intact Jurkat cells by electroporation increased effector caspase activity 25% and 18% above control, whereas no increase was observed in K562 cells. Comparable results were obtained after activating effector caspases in cytosolic lysates (data not shown).

Therefore, taken together, the data demonstrate that functionally active apoptosis-inducing proteins can be introduced into primary leukemia cells by electroporation and used to selectively activate the intrinsic or extrinsic pathways of apoptosis.

**Functional Blocks in Caspase Activation Pathways Are Common in Patients with Leukemia.** To explore whether blocks in functional status of caspase activation pathways exist among leukemia specimens, we studied AML samples (n = 24) from patients with chemosensitive disease and chemoresistant disease. The clinical characteristics of these 24 patients are shown in Table 1. Cytogenetic abnormalities were classified into low-, intermediate-, and high-risk groups, according to the criteria proposed by the Southwest Oncology Group (27). A higher proportion of high-risk cytogenetic abnormalities were found among the patients with chemoresistant disease (P = 0.04). Age at diagnosis, gender, WBC count at diagnosis, percentage of blasts, and French-American-British classification subtype did not differ significantly between the two groups. Furthermore, the efficiency of protein transduction and loss of viability after electroporation did not differ between the two groups.

The functional status of caspase activation pathways was assessed in these 24 AML specimens by introducing caspase-8 (extrinsic pathway activator) or cytochrome c (intrinsic pathway activator) by electroporation (Fig. 2). Of the 24 patients, 19 (79%) had a block in at least one caspase activation pathway. Of these 19 patients, 1 had defects confined to the intrinsic pathway, 3 had blocks restricted to the extrinsic pathway, and 15 had simultaneous blocks in both pathways. The intrinsic (cytochrome c) and extrinsic (caspase-8) pathways were simultaneously intact in only 5 of the 24 (21%) patient specimens. The integrity of caspase pathways did not correlate with efficiency of uptake of the marker protein, the percentage of cells surviving electroporation, or the percentage of blasts in the sample. To determine the reproducibility of this assay, 7 patient samples were reanalyzed in separate experiments. The increase in caspase activity above the BSA control differed by ≤7% between repeat assays.

**Simultaneous Blocks in the Intrinsic and Extrinsic Caspase Activation Pathways Correlate with Chemoresistant Disease and Decreased Patient Survival.** The functional status of the caspase activation pathways was correlated with patient responses to chemotherapy and patient survival. Simultaneous blocks in the intrinsic and extrinsic caspase activation pathways occurred in 92% of patients with chemoresistant disease (11 of 12) versus 33% of patients with chemosensitive disease (4 of 12; P = 0.005; χ² analysis). Besides simultaneous blockage of both caspase activation pathways, only high-risk cytogenetic abnormalities predicted for response to chemotherapy (P = 0.04). Age at diagnosis, gender, WBC, percentage of blasts in the sample, and French-American-British classification subtype were not predictive in this cohort of patients. In a multivariate regression analysis that included blocked caspase pathways and cytogenetics in the model, blocked caspase pathways remained a significant predictor of response to therapy (P = 0.05), whereas cytogenetic abnormalities were no longer predictive (P = 0.87). Thus, the functional integrity of the caspase activation pathways was the most important predictor of response.

The functional status of caspase activation pathways also correlated with overall patient survival. Ten of 15 patients (67%) with simultaneously blocked intrinsic and extrinsic pathways died from their disease within 16 months, compared with 2 of 9 patients (22%) with at least one intact pathway. By Kaplan-Meier analysis, the estimated overall survival at 12 months for patients with at least one intact
caspase activation pathway was 89% (95% confidence interval, 79–99%) compared with 22% (95% confidence interval, 9–35%) for patients with simultaneous blocks in both pathways (Figure 3). Using a univariate Cox model, the hazard ratio for death among patients with both caspase pathways blocked compared with patients with at least one intact pathway was 3.56 (95% confidence interval, 3.33–3.78; \( P = 0.02 \)). Likewise, simultaneously blocked pathways correlated with decreased disease-free survival.

Evidence of a Distal Block in Caspase Activation Pathways in Patients with Leukemia. The simultaneous blockage of the intrinsic and extrinsic pathways seen in AMLs could result either from two independent defects that impair each pathway separately, or it could be explained by a distal block at a point of convergence of these pathways (reviewed in Ref. 2). To distinguish between the possibilities, we electroporated Granzyme B (100 nm; Calbiochem, La Jolla, CA) into AML cells from 8 patient specimens exhibiting simultaneous blocks in both pathways. Granzyme B directly cleaves and activates many caspase-family proteases, including the downstream effector caspase-3 (28). Thus, if resistance of these AMLs to electroporation of caspase-8 and cytochrome c is because of upstream blocks in each of the major caspase activation pathways, then Granzyme B should cleave and activate caspases downstream of these blocks. For all 8 of the AML specimens tested, electroporation of Granzyme B failed to induce effector caspase activity, as measured by binding of CaspTag to active caspase-3. In contrast, electroporation of Granzyme B resulted in successful activation of effector caspases in AML specimens in which one or both pathways were intact.

Comparisons of Functional Status of Caspase Activation Pathways with Levels of Endogenous Caspase-3 and Caspase Inhibitors. The results from Granzyme B electroporation experiments suggested that AML specimens that failed to respond to both an intrinsic (cytochrome c) and an extrinsic (caspase-8) pathway stimulus had a distal defect in caspase activation. Such a phenotype could result from either reduced expression of downstream effector caspases or from elevated levels of endogenous caspase-inhibitors, such as members of the IAP family of apoptosis suppressors, which directly bind and inhibit certain effector caspases (16, 29). Levels of the IAP family member XIAP have been correlated previously with differences in chemoresponses and patient survival in AML (26). Therefore, we used immunoblotting methods to compare the levels of procaspase-3, a major effector caspase, and XIAP in AML specimens.

For these experiments, protein lysates were available from 22 of the 24 AML samples. Immunoblot data were quantified by scanning densitometry, normalized for \( \beta \)-actin, and compared with an internal reference cell line, K562 (Fig. 4). Higher levels of procaspase-3 were seen in AML specimens in which both caspase activation pathways were blocked simultaneously compared with patient specimens having at least one intact pathway. The median level of procaspase-3 in AMLs with blocked pathways was 198% (range, 4–367%) relative to the K562 internal control cell line, compared with 38% (range, 6–156%) for AMLs with an intact caspase activation pathway (\( P = 0.01 \) by the \( t \) test). The levels of XIAP were also higher among AMLs with blockage of both caspase-activation pathways, although the results did not reach statistical significance. The median level of XIAP among AMLs with at least one intact pathway was 6% (range, 0.5–43%), relative to the K562 cells reference, in contrast to a median of 26% (range, 12–81%) for AMLs with blockage of both pathways (\( P = 0.14 \) by the \( t \) test). Little or no processed (active) caspase-3 was observed in these patient specimens, regardless of whether their pathways were blocked or intact, suggesting that rates of spontaneous caspase activation do not differ. Neither caspase-3 nor XIAP protein levels (as determined by immunoblotting) correlated with clinical chemosensitivity or overall patient survival. Likewise, the ratio of XIAP:caspase-3 did not correlate with clinical outcome. Therefore, we conclude that absence of downstream effector caspases does not explain the blockage to caspase activation pathways seen in AMLs.
suggesting instead that elevations in the levels or activity of suppressors of the downstream proteases are involved.

**DISCUSSION**

In this study, we have examined the functional integrity of two major caspase activation pathways in patients with leukemia and correlated the results with clinical outcome. Cytochrome c was used to stimulate the intrinsic pathway, and caspase-8 was used to stimulate the extrinsic pathway. Blocks in caspase activation pathways were common in AML, with 79% of patient specimens displaying at least one blocked pathway. Simultaneous blockage of both pathways predicted poor response to chemotherapy and was prognostic for decreased overall patient survival.

Previous attempts to correlate individual apoptotic proteins with clinical response often yielded variable results and weak correlations (5–8, 26, 30, 31), raising questions regarding the importance of apoptosis pathways in determining patient outcome. This study supports the role of apoptosis pathways as important determinants of responses to treatment. Furthermore, the findings suggest that the overall competence of apoptosis pathways determines responses to treatment more accurately than measurements of individual apoptosis proteins. In this regard, whereas levels of caspase-inhibitor XIAP did not correlate with chemoresistant disease and simultaneous blockage of both apoptosis pathways, it is important to note that XIAP is only one of eight IAP family genes present in the human genome. Thus, whereas analysis of AML specimens having two blocked apoptosis pathways suggested a defect in distal caspase activation pathways, consistent with overexpression or hyperactivity of an inhibitor of effector caspases, measurements of the levels of multiple IAPs and other apoptosis-suppressing proteins, their post-translational modifications, and interacting partners might be required to provide information comparable with the function-based assays described here.

A number of advantages to analyzing the functional status of apoptosis pathways as opposed to levels of individual apoptosis proteins can be envisioned. First, a functional analysis simultaneously accounts for the dynamic interplay and influences of multiple molecules. Second, a functional analysis can account for the positive and negative regulation of apoptotic proteins by post-translational modification that can be difficult to discern by immunoblotting or immunohistochemistry techniques. Finally, unknown molecules that influence these pathways can be functionally accounted for, although unidentified.

It should be recognized that the electroporation approach to functional interrogation of apoptosis pathways described here has some limitations. First, we used cytochrome c as an activator of the mitochondrial-mediated caspase-activation pathway. During apoptosis, mitochondria release factors other than cytochrome c, such as SMAC/DIABLO, which also modulates caspase activation by inhibiting IAPs (32). Thus, by introducing only cytochrome c, we do not account for the influence of these other mitochondrial factors. Therefore, when introducing cytochrome c to probe the status of caspase activation, all of the regulators of this post-mitochondrial pathway (e.g., SMAC) are not interrogated by our method. Second, the specificity of the CaspaTag reagent for caspase-3 is not absolute, as caspase-8 also binds the substrate, albeit at a lower affinity. Therefore, the positive signal we observed after introducing caspase-8 may have been confounded by CaspaTag binding to caspase-8. However, if nonspecific binding was a major cofounder in our assay, then we would have expected all of the samples treated with caspase-8 to have large increases in CaspaTag, and we would expect coelectroporation of XIAP to suppress caspase-8-induced caspase activation. Thus, we believe the CaspaTag substrate accurately reflects the activity of caspases that become activated downstream of caspase-8 in our assays. Finally, in the process of thawing the frozen AML samples, cell death occurred for a subset of cells. Because the cells were subjected to a density separation before electroporation, these nonviable cells were removed before analysis. As such, there was minimal background apoptosis in unelectroporated cells and those electroporated with PE-BSA alone. However, we cannot exclude the possibility that by thawing the sample, we enriched the cell population with blasts resistant to spontaneous apoptosis. Resistance to spontaneous apoptosis is correlated with response to treatment in AML (33, 34), so the thawing of samples may have magnified the differences in caspase activation pathways between patients with chemoresistant and chemosensitive disease. Nonetheless, we were able to interrogate primary leukemia samples to understand how defects in caspase activation relate to chemoresistance.

Our study may have implications for developing optimal therapeutic strategies for the treatment of AML. Combining therapies that stimulate both caspase activation pathways may be important in the treatment of AML, as approximately half of patients with an intact activation pathway have a block in the other activation arm. Therefore, treatment strategies that focus solely on one pathway may result in high rates of resistant disease. Furthermore, for patients with chemoresistant disease, the finding of defects at a point of convergence of the intrinsic and extrinsic pathways suggests a need for agents that restore apoptosis sensitivity in distal portions of apoptosis pathways (e.g., IAP inhibitors) or therapeutic strategies that stimulate caspase-independent cell death.

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