Caspase 2 and Caspase 3 as Predictors of Complete Remission and Survival in Adults with Acute Lymphoblastic Leukemia

Stefan Faderl, Peter F. Thall, Hagop M. Kantarjian, Moshe Talpaz, David Harris, Quin Van, Miloslav Beran, Steven M. Kornblau, Sherry Pierce, and Zeev Estrov

Departments of Leukemia [S. F., H. M. K., M. B., S. M. K., S. P.], Bioimmunotherapy [M. T., D. H., Q. V., Z. E.], and Biomathematics [P. F. T.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

Dysregulation of apoptosis is an important mechanism in leukemogenesis. Caspases are cysteine proteases that play a major role in the activation of apoptotic pathways and chemotherapy-induced cell death. High levels of inactive, uncleaved caspase 2 and caspase 3 have recently been associated with poor survival in patients with acute myelogenous leukemia. We hypothesized a similarly significant role for caspase 2 and caspase 3 in patients with acute lymphoblastic leukemia. We determined levels of uncleaved caspase 2 and caspase 3 by quantitative Western blot analysis in peripheral blood samples of 45 adults with newly diagnosed ALL. We evaluated patient prognostic variables and caspase levels using multivariate logistic and Cox regression models to determine their impact on complete remission rate and overall survival probability. Levels of caspase 2 and, to a lesser degree, caspase 3 were highly associated with cytogenetic abnormalities, with lower levels in the diploid group \((P = 0.016\) and \(P = 0.10\), respectively). No association between either caspase level and the percentage of bone marrow blasts was found. A high level of caspase 3 \((>0.37\) as determined graphically\) was significantly associated with achieving complete remission \((CR; P = 0.006)\). A multivariate logistic regression analysis including age, WBC count, percentage of peripheral and marrow blasts, hemoglobin, albumin, lactate dehydrogenase, bilirubin, and creatinine determined that a high level of caspase 3 was the most significant predictor of CR \((P = 0.025,\) adjusted\), with albumin the only other significant variable \((P = 0.031)\). Caspase 2 levels were not associated with probability of CR. In a multivariate Cox model for survival, however, levels of caspase 2 above 0.37 were associated with a lower survival probability than were levels below that threshold \((P = 0.064)\). High levels of caspase 3 may have a significant effect on achieving CR. Because of the limited power \((n = 45)\) of our study, the significance of caspase 2 and caspase 3 on overall survival remains to be validated by further investigations.

INTRODUCTION

Malignant transformation of hematopoietic progenitors into leukemic cells results from aberrations in the control of cell cycle regulation and proliferation, differentiation, and the abrogation of cell suicide, or apoptosis \((1, 2)\). Apoptosis in particular refers to a biochemically regulated process of automated cell death mediated through a highly organized network of interacting proteases and their inhibitors in response to noxious stimuli from either inside or outside of the cell \((3, 4)\). Inappropriate activation or inhibition of apoptosis has been implicated in many human diseases \((5, 6)\). A series of recent studies have demonstrated that most, if not all, chemotherapeutic agents exert their tumoricidal effects by inducing apoptosis in target cells and tissues \((7–10)\).

The recently identified caspases are a rapidly expanding group of cystein proteases that have been linked to apoptosis. They are synthesized as inactive zymogens and require cleavage at specific aspartic acid residues for their activation \((11)\). Caspases were first linked to apoptosis by genetic studies of the nematode Caenorhabditis elegans and its proapoptotic protein CED-3, the sequence of which is markedly similar to that of the mammalian protein ICE\(^2\), or caspase 1 \((12–14)\). Other ICE-like proteins have since been identified, and at least 10 caspases are known to date \((15)\). Caspases play a central role as both initiators and executioners of pathways that abrogate the effect of nuclear DNA repair enzymes such as poly(ADP-ribose) polymerase and other nuclear and cytosolic proteins, thereby inducing the changes characteristic of apoptotic cell death \((16–18)\).

The caspase 2 \((ICH-1, Nedd2)\) gene was identified by screening a human cDNA library with murine Nedd2 cDNA and has been localized to chromosome 7q35 \((19, 20)\). Its mRNA is alternatively spliced into two protein species of variable length \((ICH-1L\) and ICH-1\(S\)). ICH-1\(L\) possesses proapoptotic activities, whereas ICH-1\(S\) is able to inhibit apoptosis, which suggests a dual role for caspase 2 in the regulation of apoptosis. Caspase 2 is probably activated early in the apoptotic process by cleavage into three fragments that are further processed into active subunits of \(M, 18,000\) and \(M, 21,000\) \((21)\). The caspase 3 \((CPP32, Yama, priICE)\) gene has been mapped to chromosome 4q33–
Table 1  Clinical and laboratory characteristics of patients
For each quantitative variable, the median is given, followed by the minimum and maximum in parentheses. For each qualitative variable, each category count is given, followed by the corresponding percentage in parentheses.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of patients</th>
<th>Age, years (range)</th>
<th>Male/Female (%)</th>
<th>Antecedent hematological disorder (%)</th>
<th>Hemoglobin, g/dl (range)</th>
<th>WBC count (× 10^9/liter) (range)</th>
<th>Platelet count (× 10^9/liter) (range)</th>
<th>Bone marrow blasts, % (range)</th>
<th>Peripheral blood blasts, % (range)</th>
<th>Total bilirubin, mg/dl (range)</th>
<th>Creatinine, mg/dl (range)</th>
<th>Albumin, g/dl (range)</th>
<th>Lactate dehydrogenase, IU/l (range)</th>
<th>Fibrinogen, g/dl (range)</th>
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Caspases 2 and 3 as Predictors of Remission and Survival

q35.1 (20). The protein assumes a prominent role as a downstream-acting caspase, largely responsible for proteolytic cleavage of key proteins such as poly(ADP-ribose) polymerase. It is highly expressed in cells of lymphocytic origin (22). In vitro data suggest that granzyme B, a serine protease of CTLs, can activate caspase 3 by clipping off the biologically active subunits p12 and p17 (23).

Given the significance of caspase-induced apoptosis in leukemia cells of patients with AML and their influence on prognosis, we hypothesized a similarly important involvement of these caspases in patients with ALL. Therefore, in the present study, we analyzed the levels of uncleaved caspase 2 and caspase 3 in peripheral blood samples from 45 consecutive patients with ALL. A representative blot is shown. Samples are labeled from 1 to 9. Lane 1 is a normal control with the remaining lanes being samples from patients. The lane loaded with protein from K562 cells is labeled as such.

**MATERIALS AND METHODS**

**Patients and Samples.** Peripheral blood samples from 45 consecutive patients with ALL were obtained before the start of therapy as part of protocols approved by the Human Subjects Committee of The University of Texas M. D. Anderson Cancer Center. Samples were obtained with the informed consent of the patients. Low-density cell fractions were immediately obtained by Ficoll-Hypaque gradient centrifugation from these samples and then frozen for further processing. Peripheral blood cells from four healthy volunteers were used as controls. The clinical and laboratory characteristics of the patients whose samples were used in this study are summarized in Table 1.

A diagnosis of ALL was based on morphological analysis of peripheral blood and bone marrow aspirates, and biopsies according to guidelines published by the French-American-British Cooperative Group (25). Diagnostic evaluations included cytochemical staining with myeloperoxidase, terminal deoxynucleotidyl transferase, esterase stains, and periodic acid-Schiff, and immunophenotyping by flow cytometry. ALL was diagnosed if blasts were morphologically lymphoid, terminal deoxynucleotidyl transferase positive and myeloperoxidase-negative, and positive for at least two T-cell markers, or for CD19, CD20, or CD10 (common acute lymphocytic leukemia antigen).

Thirty-six patients (80%) received induction therapy with hyper-CVAD. This treatment consisted of eight intensive chemotherapy courses alternating between hyper-CVAD (300 mg/m² cyclophosphamide i.v. every 12 h × six doses on days 1 to 3, 2 mg vincristine i.v. on days 4 and 11, 50 mg/m² doxorubicin i.v. on day 4, and 40 mg/day dexamethasone i.v. or p.o. days 1–4 and 11–14) during courses 1, 3, 5, and 7 and methotrexate/cytarabine (1 g/m² methotrexate on day 1, 3 g/m² cytarabine every 12 h × four doses on days 2 and 3) during courses 2, 4, 6, and 8. Patients ≥60 years received a reduced dose of cytarabine at 1 g/m². Maintenance therapy consisted of POMP (methotrexate, 6-mercaptopurine, vincristine, and prednisone) and two courses of polyethylene-glycol-asparaginase with etoposide as described elsewhere (26).

**Cytogenetic Analysis.** Cytogenetic studies were performed as described previously (27). The patients were classified into three cytogenetic categories: diploid, Ph chromosome-positive, and other (hyperdiploid, hypodiploid, and pseudodiploid; Table 1). There were too few patients in the “other” category to warrant classification of a separate entity.

**Western Immunoblotting.** Low-density cells from peripheral blood samples were obtained by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) fractionation. Western immunoblotting was performed on cell lysates as described previously (24). Each gel run included a sample of K562 cells (American Type Culture Collection, Rockville, MD), molecular weight markers, and two samples of peripheral blood cells from healthy donors.
The following antibodies were used for detection of the respective proteins: monoclonal mouse antihuman ICH-1 L (caspase 2), mouse antihuman CPP32 (caspase 3; Transduction Laboratories, Lexington, KY), and mouse antihuman tubulin (Sigma Chemical Co., St. Louis, MO). Normal mouse IgG (Sigma Chemical Co.) was used as a control. Bound antibody was detected according to the ECL protocol (Amersham Life Science, Arlington Heights, IL). Chemiluminescence was detected on X-OMAT AR5 film (Eastman Kodak, Rochester, NY). The levels of caspases 2 and 3 were scored on a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA). The results were normalized by dividing the numerical value of a sample’s caspase 2 or caspase 3 signal by the signal of the same caspase obtained from the K562-positive control on the same blot as described previously (24). To ensure standardization of the positive control, all K562 cell protein came from a single, large protein preparation aliquoted into a single-use vial as described previously (28). A representative immunoblot is shown in Fig. 1.

**Statistical Methods.** Associations between patient covariates were assessed graphically for pairs of numerical variables by examining scatterplots, by Wilcoxon-Mann-Whitney and Kruskal-Wallis test statistics for categorical and continuous variables, and by the Fisher exact test and its generalizations for pairs of categorical variables (29–31). Logistic regression was used to assess the ability of covariates to predict the probability of CR. A multivariate logistic regression model was obtained by performing a backward elimination with a $P$ cutoff of 0.05, and then allowing any variable previously deleted to reenter the final model if its $P$ was $<0.05$. Goodness-of-fit was assessed by residual and partial residual scatterplots and likelihood ratio statistics. Unadjusted survival and EFS analyses were performed using Kaplan-Meier plots (32). Unadjusted comparisons of survival and EFS between patient subgroups were made using the log-rank test (33). The Cox proportional hazards model and its generalizations were used to assess the ability of the caspases and patient characteristics to predict survival and EFS (34, 35). Goodness-of-fit was assessed by the Grambsch-Therneau test.

**Table 2** Association between caspase 2 and 3 level and cytogenetic groups

<table>
<thead>
<tr>
<th>Cytogenetic group</th>
<th>Median</th>
<th>Range</th>
<th>$P^a$</th>
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</thead>
<tbody>
<tr>
<td>Caspase 2</td>
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<tr>
<td>Diploid</td>
<td>0.19</td>
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<td>0.016</td>
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<td>Ph+</td>
<td>0.21</td>
<td>0.04–0.83</td>
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<tr>
<td>Other</td>
<td>0.36</td>
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<tr>
<td>Caspase 3</td>
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<tr>
<td>Diploid</td>
<td>0.205</td>
<td>0.009–0.62</td>
<td>0.10</td>
</tr>
<tr>
<td>Ph+</td>
<td>0.14</td>
<td>0.005–0.72</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>0.4</td>
<td>0.03–4.14</td>
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</table>

$^a$Kruskal-Wallis test (two-sided).

**Fig. 3** A threshold level of $\geq 0.37$ for caspase 3 levels is highly associated with CR. All 19 patients above this level achieved CR, compared with only 17 of 26 (65%) patients below this level.
Schoenfeld residual plots, and Martingale residual plots (35, 36). Scatterplots were smoothed using the locally weighted regression smoothing method by Cleveland (37). The P for assessing the effect of caspase 3 dichotomized at an optimal cutpoint was adjusted using the method of Lausen and Schumacher (38) and Hilsenbeck and Clark (39). All computations were carried out on a DEC Alpha 2100 5/250 system computer (Digital Electronics Corporation, Nashua, NH) in Splus and StatXact (Cytel Software Corporation) using both standard Splus functions and the Splus survival analysis package of Therneau (40, 41).

RESULTS

Levels of Caspases 2 and 3 in Peripheral Blood of ALL Patients and Normal Controls. Peripheral blood specimens from 45 patients with newly diagnosed ALL were analyzed to quantitate the levels of caspase 2 and caspase 3 (Fig. 1). Specimens were obtained from newly presenting patients as well as from stored, frozen samples from other participating laboratories at M. D. Anderson Cancer Center. Patient characteristics are summarized in Table 1. The unequal distribution of male and female patients was attributable to the availability of patient material at the time of testing and not to an intended selection process. Normal controls were obtained from the peripheral blood of healthy volunteers. The median normalized values in normal controls were 0.06 (range, 0.03–0.13) for caspase 2 and 0.04 (range, 0.03–0.13) for caspase 3. Median values were lower in normal controls than in patient samples.

Association of Caspase 2 and 3 Levels with Cytogenetic Abnormalities, Bone Marrow Blasts, and Hemoglobin. The majority of cases of ALL exhibited karyotype abnormalities (39). Because some of these cytogenetic aberrations such as translocation t(9;22)(q34;q11) are associated with poor outcome, we explored whether levels of caspase 2 and caspase 3 were associated with cytogenetics using the two-sided Kruskal-Wallis test (Fig. 2; Table 2). We found a significant association between normalized levels of caspase 2 and cytogenetic group (P = 0.016). The lowest median values of caspase 2 levels were found in diploid patients (P = 0.19) and in Ph-positive patients with ALL (P = 0.21), whereas patients with other cytogenetic abnormalities had much higher levels (P = 0.36), with the variability in caspase 2 greatest in the “other” category, as might be expected. The association between caspase 3 and cytogenetic abnormalities was weaker (P = 0.10) but followed the same distribution pattern as for caspase 2. Diploid patients had median caspase 3 levels that were between those for patients with Ph-positive ALL and those for patients with other karyotype abnormalities. No association was found between the normalized levels of caspases 2 and 3 and bone marrow blasts or hemoglobin levels.

Association of Caspase 2 and 3 Levels with CR Rate.

The overall CR rate among the 45 patients in this study was 80% (36 of 45). No association was found between gender and CR or between cytogenetic profile and CR.

A plot of CR (1 = yes, 0 = no) on caspase 3 (Fig. 3) showed a striking relationship between caspase 3 level and CR. Specifically, 17 of 26 patients with caspase 3 levels of ≤0.37 (65%) achieved CR, whereas all 19 patients with caspase 3 levels >0.37 achieved CR (P = 0.006; Table 3). The Fisher exact P for this 2 × 2 cross classification is 0.0058. When adjusted to account for the optimal cutpoint search, it increases to P = 0.20 which is still significant. There was no similar threshold effect for caspase 2 level and CR.

Because of this apparent strong threshold effect, we used the binary indicator of whether caspase 3 was >0.37, rather than its numerical value, in the multivariate logistic regression analysis. In addition to normalized levels of caspase 2 and caspase 3, we evaluated the prognostic impact of age, WBC count, percent peripheral and bone marrow blasts, hemoglobin, albumin, lactate dehydrogenase, bilirubin, and creatinine on the probability of CR. A multivariate logistic regression model was fit starting with all variables that were prognostic in univariate analyses. The final model included only albumin and a high level of caspase 3 (>0.37). A high level of caspase 3 was the most significant predictor of CR (P = 0.025, adjusted; Table 4).

Association of Caspase 2 and 3 Levels with Survival.

Twenty-two patients died during a follow-up period of 63 months. The median survival was 165 weeks, with a 95% lower confidence bound of 90 weeks. Survival according to levels of caspase 2 above and below 0.37 is shown in Fig. 4.
Hemoglobin and albumin levels were highly associated with survival. Hemoglobin values of 7–10 g/dl were associated with a higher risk of death, and values >10 g/dl with an increasingly lower risk (because of a “cubic” relationship on the log hazard scale). Albumin values <3.5 g/dl were associated with a higher risk of death, and values >3.5 were associated with a lower risk (because of a “quadratic” relationship on the log hazard scale). These nonlinear cubic and quadratic relationships were determined via preliminary goodness-of-fit analyses, including Martingale residual plots and tests of parametric significance.

Platelets and the percentage of bone marrow blasts were highly negatively associated (Spearman correlation, -0.37). Therefore, because of collinearity, neither was a significant predictor of survival when both were included in the multivariate Cox model for survival. When platelets were replaced by bone marrow blasts in the final survival model, bone marrow blasts have a P of 0.086. Thus, either platelets or percentage of bone marrow blasts were marginally significant predictors of survival after accounting for the other covariates in the model.

Because of the strong association between levels of uncleaved caspases 2 and 3 (Fig. 5) and to avoid collinearity, no model was fit that included both of these variables. After accounting for the other covariates in the model, caspase 3 level was not predictive of survival (P = 0.24). However, the binary indicator that caspase 2 was 0.37 or larger was marginally significant (P = 0.064). A higher risk of death was associated with caspase 2 levels above 0.37, as indicated by the solid nonparametric regression line in Fig. 6. Again, because of the small sample size, this effect remains to be validated by future studies. The solid line (caspase 2) and dashed line (caspase 3) each are smoothed versions of the Martingale residual scatter-plot. Each line visually represents the manner in which the risk of death varies as a function of that variable, with values >0 corresponding to a higher risk of death, and values <0 to a lower risk of death. The final multivariate Cox model is summarized in Table 5.

### DISCUSSION

Apoptosis describes an energy-dependent, organized process of noninflammatory cell death by which tissues rid themselves of cells that have sustained irreparable damage, such as that attributable to exposure to radiation or chemotherapeutic agents. Caspases are ubiquitously distributed cysteine proteases that are considered to be the executioners of apoptotic cell death. Once they have been activated by proteolytic cleavage from inactive zymogens, the cell proceeds with the breakdown of essential nuclear and cytosolic proteins that inevitably results in the formation of characteristic apoptotic bodies and the cell’s demise (11, 43). We have hypothesized that aberrant caspase pathways in leukemic cells of patients with ALL fail to respond to apoptotic stimuli and become resistant to treatment with cytotoxic drugs, therefore worsening clinical outcome. If true, then lack of caspase cleavage would thus lead to increased intracellular levels, which in turn could predict clinical outcome.

The sample size studied was small (n = 45), attributable in large part to the fact that adult ALL is a rare disease. Consequently, there is limited power to detect associations between the two caspase variables and patient outcome. Nonetheless, we were able to demonstrate in a multivariate logistic regression

**Table 5** Final multivariate Cox model for prediction of survival

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<td>Performance status</td>
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<td>Hemoglobin</td>
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<td>Albumin</td>
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<td>Caspase 2</td>
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<td>Caspase 3</td>
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analysis a critical cutoff level for normalized caspase 3 (>0.37) above which the probability of achieving a CR was significantly higher. We did not find a similar threshold level for caspase 2. Interestingly, however, caspase 2 levels above the same threshold level of 0.37 showed a trend for lower survival probability, as suggested by a multivariate Cox model of survival.

Together, these results partly confirmed the findings of our previous study in which high levels of both caspase 2 and caspase 3 were associated with poor EFS and overall survival in patients with AML (24). Instead of finding low levels of caspases as might be expected in cells unable to undergo apoptosis because of defects in the caspase pathway, we found high levels of both uncleaved caspases. We also demonstrated that lack of cleavage and accumulation of inactive caspases accounts for the higher caspase levels and worse outcome in AML patients. The same mechanisms may be operative in ALL. It is possible that the elevated levels of caspases we observed originated from cells other than leukemic blasts and that their variations in the leukemia cells may have been too small to be significant. However, the chromosomal location of the genes for the caspases was not involved in any cytogenetic rearrangements that caused overexpression of caspases in any of the samples tested. Furthermore, caspase levels in T lymphocytes, which make up most of the normal peripheral blood low-density cells, were found to be lower than in leukemic cells (24). These results are supported by data from other investigators who found low levels of caspase 3 in normal hematopoietic progenitors (44).

Is it plausible that caspase 2 and 3 might have different effects on CR and survival in patients with ALL? (a) Our small sample size may have precluded us from detecting significant effects of both caspases on both probability of CR and probability of survival; and (b) the caspase apoptotic pathway constitutes a complex network of interacting proteases and their inhibitors. Individual caspases may demonstrate tissue, substrate, or stimulus specificity. Caspases may be localized in specific cellular compartments that allow cleavage and activation only under well-defined circumstances and triggers (45). Caspases have been divided into various categories based on the length of their NH₂-terminal domains (10). Caspases with long prodomains, such as caspase 2, act further upstream in the cascade as initiators or regulators and may well be overlapped by other competing modulating pathways, whereas caspases with shorter prodomains, particularly caspase 3, act downstream at a bottleneck, where most of the apoptotic signals merge and execution of apoptosis is imminent (46). This may explain the different effects of caspase 2 and caspase 3 levels on CR and survival in our study.

How can we explain why high levels of caspases may confer a lower probability of survival? Similarly, is it plausible to infer that a high level of either caspase confers a lower probability of survival? Leukemic cells may respond to cytotoxic agents only if they express a minimal level of caspases. The ability of the cell to attain these levels will subsequently influence the overall response to therapeutic interventions as reflected, for example, by the CR rate and survival probability. In AML, we found high levels of the uncleaved caspases 2 and 3. However, caspase 3 could not be cleaved spontaneously in most cases. Therefore, despite high intracellular levels of pro-apoptotic enzymes, the host was unable to eradicate residual disease after therapy with cytotoxic agents, and the overall survival of patients decreased (24).

In summary, our data suggest that levels of caspase 3 above a critical threshold are strikingly related to the probability of achieving CR, whereas caspase 2 levels are not. However, elevated levels of caspase 2 may negatively influence survival of ALL patients, much as they do in AML patients. Further studies are now required to analyze the levels of cleaved and uncleaved caspases and their target products in lymphoblasts as well as other pathways that may be incriminated in the activation or defective operation of apoptosis in these cells.

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Stefan Faderl, Peter F. Thall, Hagop M. Kantarjian, et al.


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