Check for updates

Brief report

Intact apoptosis signaling in myeloid leukemia cells determines treatment outcome in childhood AML

Lüder H. Meyer,[1] Manon Queudeville,[1] Sarah M. Eckhoff,[1] Ursula Creutzig,[2] Dirk Reinhardt,[3] Leonid Karawajew,[4] Wolf-Dieter Ludwig,[4] Karsten Stahnke,[1] and Klaus-Michael Debatin[1]

[1]Department of Paediatrics and Adolescent Medicine, University of Ulm, Ulm; [2]Paediatric Haematology and Oncology, University of Münster, Münster; [3]Paediatric Haematology and Oncology, Medical School Hannover, Hannover; and [4]Department of Haematology, Oncology and Tumor Immunology, Robert-Roessle-Clinic at the HELIOS Klinikum Berlin, Charité Medical School, Berlin, Germany

Recently we reported that intact apoptosis signaling is indicative of favorable outcome in childhood acute lymphoblastic leukemia. Here we addressed this issue in 45 pediatric acute myeloid leukemia patients analyzing 2 core apoptogenic events: cytochrome c release and caspase-3 activation. In patients with good prognosis cytochrome c release was clearly found to be caspase-dependent and correlated with activated caspase-3, indicating that activation of initiator or amplifier caspases such as caspase-8 together with an intact apoptosome function are elementary for favorable outcome. The functional integrity of this apoptogenic checkpoint is reflected by the parameter caspase-dependent cytochrome c-related activation of caspase-3 ($CRAC^{dep}$). Patients with positive $CRAC^{dep}$ values (intact signaling) exhibited superior survival compared with $CRAC^{dep}$ negative patients (deficient signaling). Thus, the propensity to undergo apoptosis of leukemia cells is an important feature for favorable treatment outcome and may serve as an additional stratification tool for pediatric AML patients. This trial was registered at www.ClinicalTrials.gov as #NCT00111345. (Blood. 2008;111:2899-2903)

© 2008 by The American Society of Hematology

Introduction

Successful treatment of children with acute myeloid leukemia (AML) has improved, reaching overall survival rates of 62%.[1] A considerable number of patients still encounter relapse, however. Because apoptosis plays a key role in regulating tissue homeostasis, defects in apoptosis signaling have been considered to be responsible for treatment failure. Consequently, several apoptosis-regulating molecules have been evaluated for their prognostic impact. Imbalanced expression of Bax and Bcl-2 in favor of the proapoptotic molecule was found to be correlated to good prognosis in AML.[2] On the contrary, studies assessing transcript levels of apoptosis signaling molecules showed inverse findings.[3] However, expression of single factors may not reflect the efficiency of the cell death machinery. For this reason we previously developed a different approach analyzing the functional integrity of apoptosis signaling in individual cells by simultaneously investigating 2 key apoptogenic events: mitochondrial release of cytochrome c and activation of caspase-3.[4] Using this method, a prognostic potential of intact apoptosis signaling in acute lymphoblastic leukemia (ALL) cells was described. A new parameter, cytochrome c–related activation of caspases (CRAC), was identified indicating intact or defective cytochrome c–related activation of caspases and implying prognostic impact.[5] Based on these findings we analyzed 45 pediatric AML cell samples for the significance of intact apoptosis signaling for successful treatment.

Methods

The AML Berlin-Frankfurt-Münster (BFM) study was approved by the ethics commission of the board of physicians in Westfalen-Lippe, Germany, and the ethics review board of the faculty of medicine, University of Münster, Germany. Analysis of patient material was carried out in accordance with the guidelines of the AML BFM study.

Forty five diagnostic samples (frozen bone marrow or peripheral blood) obtained before treatment from pediatric de novo AML patients were analyzed. Patients were treated according to the AML BFM-98 or -2004 protocols after informed consent was obtained in accordance with the Declaration of Helsinki. Treatment and diagnosis of relapse were accomplished according to the AML BFM trial criteria. Treatment response or complete remission (CR) were examined by bone marrow morphology (BM) and detection of minimal residual disease (MRD) by flow cytometry (time point [tp] 1, day 15; tp2, days 21-28; tp3, days 42-56; and tp4, days 70-84 of treatment) as described earlier.[1,6] Leukemia cells were analyzed for apoptosis signaling and correlated to clinical data as described previously.[5] In brief, after induction of spontaneous apoptosis by factor deprivation in culture[7] (with or without 100 μM zVADfmk), cells were analyzed by flow cytometry and evaluated in the caspase-3 versus cytochrome c plot. The parameters cell death, active caspase-3 (ac), total cytochrome c release (cc^{total}), caspase-dependent (cc^{dep}) and -independent (cc^{indep}) cytochrome c release were quantified as described earlier[5] (Figure 1A).

Submitted August 24, 2007; accepted November 22, 2007. Prepublished online as *Blood* First Edition paper, December 14, 2007; DOI 10.1182/blood-2007-08-109058.

K.S. and K.-M.D. contributed equally to this paper.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2008 by The American Society of Hematology

Downloaded from http://ashpublications.org/blood/article-pdf/111/5/2899/486868/zh800508002899.pdf by guest on 12 December 2024

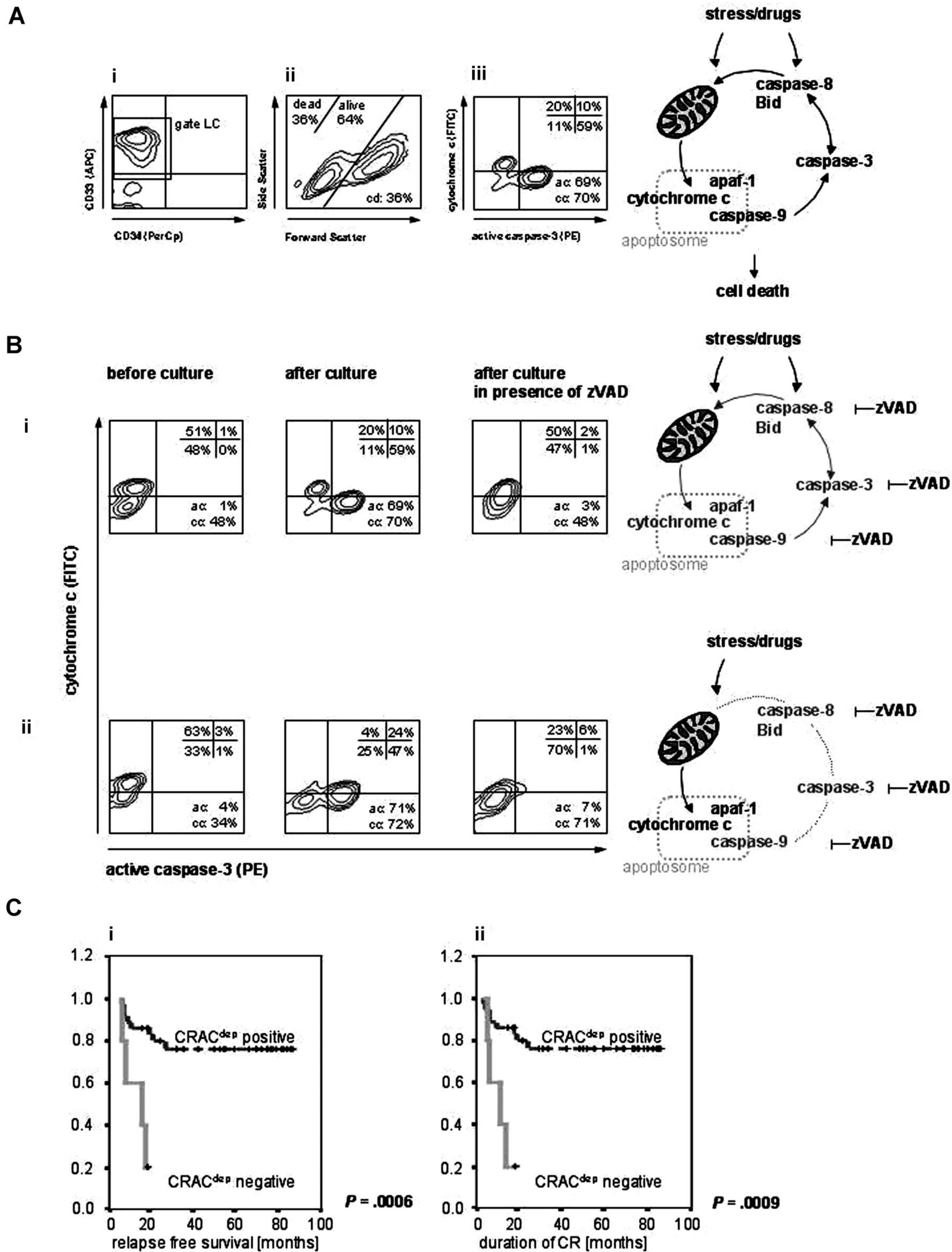

Figure 1. Quantification of apoptosis events in myeloid leukemia cells. Leukemia cells from pediatric AML patients were analyzed before and after culture with or without the caspase inhibitor zVADfmk by flow cytometry. (A) Identification of leukemia cells and analysis of cell death parameters. Leukemia cells were identified by surface markers (i: CD33 or CD34, gate LC) and further analysis was conducted gated on these cells. Cell death (cd) was estimated by forward/side scatter profile principally detecting apoptotic cells by changes of decreased volume and increased light scattering (ii). Activated caspase-3 (ac) and cytochrome c release (cc) were analyzed by simultaneous intracellular staining (phycoerythrin (PE)-conjugated anti–active caspase-3 and anti–cytochrome c 7H8.2C12 antibody, the latter followed by fluorescein-isothiocyanate (FITC)-conjugated goat anti–mouse IgG2b antibody) and quantified in the ac versus cc plot (iii). The staining was established using isotype-matched fluorochrome-conjugated unspecific antibodies. Cells with active caspase-3 were quantified as percentage of events in the upper and lower right quadrants (ac), cytochrome c release was estimated counting events in the lower left and right quadrants (cc). Because caspase-3 activation is completely caspase dependent, only the zVAD inhibitable caspase activation was considered (ac: difference of ac values after culture with and without zVAD); caspase dependent cytochrome c release (cc^{dep}: difference of cc values after culture with and without zVAD) was discriminated from independent (cc^{indep}: difference of cc values before and after culture with zVAD) and total cytochrome c release (cc^{total}, sum of cc^{dep} and cc^{indep}). (B) Distinct patterns of apoptosis signaling. Cytochrome c versus active caspase-3 plots for 2 patient samples showing cytochrome c release dependent (i) or independent (ii) on upstream or amplifier caspases such as caspase-8, resulting in positive (i) or negative (ii) $CRAC^{dep}$ values. (C) Treatment outcome in different patient groups according to $CRAC^{dep}$. Superior relapse-free survival (i) and superior continuing remission (ii) of $CRAC^{dep}$-positive pediatric AML patients. Kaplan Meier analysis, *P* indicates significance.

Downloaded from http://ashpublications.org/blood/article-pdf/111/5/2899/486868/zh800508002899.pdf by guest on 12 December 2024

Results and discussion

Release of cytochrome c and consecutive apoptosome formation lead to activation of downstream effector caspases such as caspase-3, resulting in cells staining low for cytochrome c and positive for activated caspase-3 (Figure 1Bi,ii). Apoptosomal dysfunction results in impaired caspase-3 activation despite release of cytochrome c. Caspases other than caspase-3 acting upstream of mitochondria, such as caspase-8, are also involved in initiating or amplifying the formation or activity of this complex. By incubation in the presence or absence of the pan-caspase inhibitor zVADfmk, cytochrome c release (cc) was observed to be dependent (Figure 1Bi) or independent (Figure 1Bii) on upstream or amplifier caspases (cc^{dep}, cc^{indep}). Evaluating the differences in signaling pattern with respect to treatment outcome, the absolute values for cell death (cd), active caspase-3 (ac), and released cytochrome c (cc^{total}, cc^{dep} and cc^{indep}, respectively) were compared (independent samples *t* test) in patients grouped according to treatment response. No differences were found for the mean values of cd, cc^{total} and cc^{indep}. However, differences in mean values for cc^{dep} were detected comparing patients with good or poor response to treatment (BM; tp1: $P = .036$, tp3: $P = .014$ and CR/no CR: $P = .016$). Higher values for ac were observed in patients encountering relapse (overall relapse, trend $P = .057$; relapse within 12 months, $P = .025$ and within 24 months, $P = .015$) in contrast to not or late relapsing patients. According to the AML BFM protocol, patients are stratified into standard risk (SR) or high risk (HR) groups based on French-American-British (FAB) classification, cytogenetics, and response to induction therapy on day 15. Although in patient groups differentially responding on day 15 to induction therapy, no difference in ac values was found; HR patients showed higher ac values than SR patients ($P = .002$). Active caspase-3 was identified as an important factor for outcome in pediatric ALL and AML, however, with opposite features. While ALL patients with good response and no relapse revealed high levels of caspase activation, AML patients displayed an association of high caspase-3 activation to high risk and relapse. Likewise, in adult ALL high caspase-3 expression correlated with complete remission,[8] whereas high levels of activated caspase-3 were associated with impaired apoptosis and decreased survival of adult AML patients.[9] Together with conflicting reports on the prognostic relevance of caspase-3 in leukemia,[10-12] these findings strongly suggest that the complex regulated apoptosis signaling system is insufficiently represented if analyzed by one molecule alone. The findings also emphasise the importance to analyze checkpoints or molecules integrating diverse apoptosis signaling pathways, such as cytochrome c, caspase-3, and apoptosome formation.

Table 1. Correlation of active caspase-3 to cytochrome c release, caspase dependent (ac to cc^{dep})

	n	r_s	*P*
Total	45	0.360*	.015
tp1, BM blast cells			
Less than 5%	28	0.568*	.002
Greater than 5%	9	0.548	.127
tp1, MRD (flow cytometry)			
Negative	13	0.642*	.018
Positive	23	0.358	.094
tp2, BM blast cells			
Less than 5%	14	0.646*	.043
Greater than 5%	21	0.287	.124
tp2, MRD (flow cytometry)			
Negative	14	0.528*	.014
Positive	21	0.055	.851
tp3, BM blast cells			
Less than 5%	26	0.619*	.006
Greater than 5%	14	-0.094	.750
tp3, MRD (flow cytometry)			
Negative	8	0.764*	.027
Positive	12	0.267	.402
tp4, BM blast cells			
Less than 5%	36	0.497*	.002
Greater than 5%	4	-0.258	.742
tp4, MRD (flow cytometry)			
Negative	23	0.386	.069
Positive	7	-0.204	.661
CR			
Yes	40	0.352*	.026
No	5	0.0	.000
Duration of CR, (solely CR patients)			
More than 1 year	32	0.460*	.008
Less than 1year	8	-0.074	.862
Relapse			
No	28	0.519*	.005
Yes	12	0.134	.679
Relapse within 24 months after diagnosis			
No	29	0.516*	.004
Yes	11	0.070	.838

r_s indicates correlation coefficient; and *P*, significance.
*Correlation is significant (2-tailed).

We hypothesised that intact apoptosis signaling is characterized by concomitant release of cytochrome c and caspase-3 activation and therefore would be found in the patient groups with favorable outcome. Correlating these 2 events directly

Table 2. Relapse-free survival in different risk groups according to $CRAC^{dep}$

	No. total	No. censored	No. of events (=relapse)	Relapse-free survival, mo	SE	CI	*P*
All							
$CRAC^{dep}$ positive	35	27	8	69.0	5.3	58.6-79.5	<.001
$CRAC^{dep}$ negative	5	1	4	13.3	2.2	8.9-17.6	—
SR							
$CRAC^{dep}$ positive	10	9	1	79.1	6.7	66.0-92.2	—
$CRAC^{dep}$ negative	—	—	—	—	—	—	—
HR							
$CRAC^{dep}$ positive	24	17	7	63.4	6.9	49.7-77.0	.010
$CRAC^{dep}$ negative	5	1	4	13.3	2.2	8.9-17.6	—

CI indicates 95% confidence interval; and —, not applicable.

Downloaded from http://ashpublications.org/blood/article-pdf/111/5/2899/486868/zh800508002899.pdf by guest on 12 December 2024

Table 3. Duration of complete remission (CR) in different risk groups according to CRAC^{dep}

Treatment groups	No. total	No. censored	No. of events (=relapse)	Duration of CR, mo	SE	CI	P
All							
CRAC^{dep} positive	35	27	8	68.5	5.4	58.8-79.1	.001
CRAC^{dep} negative	5	1	4	11.4	2.2	7.1-15.7	—
SR							
CRAC^{dep} positive	10	9	1	78.7	6.9	65.3-92.2	—
CRAC^{dep} negative	—	—	—	—	—	—	—
HR							
CRAC^{dep} positive	24	17	7	62.6	7.1	48.7-76.6	.014
CRAC^{dep} negative	5	1	4	11.4	2.2	7.1-15.7	—

Risk group assignment was not available for one patient (CRAC^{dep} positive and no relapse) in these analyses.
P indicates significance by log rank test; and —, not applicable.

revealed that only cytochrome c release dependent on amplifying caspases (cc^{dep}) significantly correlated with activated caspase-3 and was exclusively detected in patient groups with good response to initial treatment (BM blasts $< 5\%$, tp1-4) or groups exhibiting MRD negativity (tp1-3, tp4 trend) (Table 1). The same finding was made in the groups achieving CR or retaining CR longer than 1 year and in the group without relapse. Remarkably, this correlation was completely absent in the group with poor response to initial treatment, groups not reaching CR or with CR less than 1 year as well as in the group of relapsing patients. This indicates that an intact apoptosome function and active amplifying caspases are elementary for favorable outcome in childhood AML.

The functional integrity of this important apoptogenic checkpoint is subsumed by the parameter "caspase dependent cytochrome c-related activation of caspase-3" (CRAC^{dep}), which was calculated as the difference of ac and cc^{dep} in equivalence to the CRAC-value described for ALL.[5] Proficient signaling results in positive CRAC^{dep} values in contrast to deficient signaling resulting in negative CRAC^{dep} values. Division of the patient cohort according to this new parameter resulted in 38 CRAC^{dep}-positive and 7 -negative patients. The distribution of CRAC^{dep} values was unrelated to features such as gender, FAB subtype, age or initial blast cell count (Table S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Maintenance of CR longer than 1 year predisposes for favorable outcome in pediatric AML.[13,14] Thirty of 32 patients sustaining CR for more than 1 year were CRAC^{dep}-positive (Fisher exact test, $P = .015$). Analysis of survival and remission duration revealed a superior outcome for CRAC^{dep}-positive patients overall but also specifically in the HR patient group. Notably, SR stratified patients merely displayed CRAC^{dep}-positivity (Figure 1C, Tables 2,3). Multivariate analysis revealed an increased risk for relapse and decreased probability of continuous CR for CRAC^{dep} negative patients in contrast to established risk factors like day 15 remission failure or hyperleucocytosis[15] (Table 4).

Taken together, we clearly found that the propensity to undergo apoptosis of leukemia cells delineated by intact cytochrome c related caspase-3 activation (CRAC^{dep}) is required for successful treatment of pediatric AML. Analysis of the functional integrity of this apoptosis checkpoint can thus be used for future treatment stratification.

Acknowledgments

We thank Johanna Pfeil for her excellent technical assistance.

This work was supported by a research fellowship of the European Hematology Association (L.H.M.) and a grant of the Deutsche Forschungsgemeinschaft (K.S. and K.-M. D.).

Authorship

Contribution: L.H.M. designed research, performed experiments, analyzed data, and wrote the manuscript; M.Q. performed experiments, analyzed data, prepared the figures, and critically read the manuscript; S.M.E. analyzed data and critically read the manuscript; U.C., D.R., and W.-D.L. collected and provided patient samples and clinical data, and critically read the manuscript; K.S. and L.K. designed research and critically read the manuscript; and K.-M.D. designed research, analyzed data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Klaus-Michael Debatin, University of Ulm, Department of Paediatrics and Adolescent Medicine, Eythstrasse 24, 89075 Ulm, Germany; e-mail: klaus-michael.debatin@uniklinik-ulm.de.

Table 4. Multivariate analyses of risk ratio for relapse or continuous complete remission (CCR) using the Cox regression model

	Risk ratio			
	Relapse	CCR	CI	P
CRAC^{dep} negativity	6.08	—	1.21-30.52	.028
Failure of remission on day 15	3.41	—	0.85-13.69	.083
Hyperleucocytosis (WBC $> 100 \times 10^9$/L)	2.09	—	0.53-8.21	.293
CRAC^{dep} negativity	—	.15	0.03-0.75	.020
Failure of remission on day 15	—	.24	0.06-0.91	.036
Hyperleucocytosis (WBC $> 100 \times 10^9$/L)	—	.45	0.11-1.84	.264

Number of patients was 35 (day 15 remission status was unknown for 5 of 40 patients who achieved remission).
WBC indicates white blood cell count; CCR, continuous complete remission; and —, not applicable.

Downloaded from http://ashpublications.org/blood/article-pdf/111/5/2899/486868/zh800508002899.pdf by guest on 12 December 2024

References

1. Creutzig U, Zimmermann M, Lehrnbecher T, et al. Less toxicity by optimizing chemotherapy, but not by addition of granulocyte colony-stimulating factor in children and adolescents with acute myeloid leukemia: results of AML-BFM 98. J Clin Oncol. 2006;24:4499-4506.
2. Del Poeta G, Venditti A, Del Principe MI, et al. Amount of spontaneous apoptosis detected by Bax/Bcl-2 ratio predicts outcome in acute myeloid leukemia (AML). Blood. 2003;101:2125-2131.
3. Hess C, Berkhof J, Denkers F, et al. Activated intrinsic apoptosis pathway is a key related prognostic parameter in acute myeloid leukemia. J Clin Oncol. 2007;25:1209-1215.
4. Stahnke K, Mohr A, Liu J, Meyer LH, Karawajew L, Debatin KM. Identification of deficient mitochondrial signaling in apoptosis resistant leukemia cells by flow cytometric analysis of intracellular cytochrome c, caspase-3 and apoptosis. Apoptosis. 2004;9:457-465.
5. Meyer LH, Karawajew L, Schrappe M, Ludwig WD, Debatin KM, Stahnke K. Cytochrome c related caspase-3 activation determines treatment response and relapse in childhood B-precursor ALL. Blood. 2006;107:4524-4531.
6. Langebrake C, Creutzig U, Dworzak M, et al. Residual disease monitoring in childhood acute myeloid leukemia by multiparameter flow cytometry: the MRD-AML-BFM Study Group. J Clin Oncol. 2006;24:3686-3692.
7. Ryningen A, Wergeland L, Glenjen N, Gjertsen B, Bruserud O. In vitro crosstalk between fibroblasts and native human acute myelogenous leukemia (AML) blasts via local cytokine networks results in increased proliferation and decreased apoptosis of AML cells as well as increased levels of proangiogenic Interleukin 8. Leuk Res. 2004;29:185-196.
8. Faderl S, Thall P, Kantarjian H, et al. Caspase 2 and caspase 3 as predictors of complete remission and survival in adults with acute lymphoblastic leukemia. Clin Cancer Res. 1999;5:4041-4007.
9. Oliver L, Vavasseur F, Mahe B, et al. Assessment of caspase activity as a possible prognostic factor in acute myeloid leukaemia. Brit J Haematol. 2002;118:434-437.
10. Estrov Z, Thall PF, Talpaz M, et al. Caspase 2 and caspase 3 protein levels as predictors of survival in acute myelogenous leukemia. Blood. 1998;92: 3090-3097.
11. Svingen PA, Karp JE, Krajewski S, et al. Evaluation of Apaf-1 and procaspases-2, -3, -7, -8, and -9 as potential prognostic markers in acute leukemia. Blood. 2000;96:3922-3931.
12. Gronda M, Brandwein J, Minden M, et al. Assessment of the downstream portion of the mitochondrial pathway of caspase activation in patients with acute myeloid leukemia. Apoptosis. 2005;10: 1285-1294.
13. Castellino S, Alonzo T, Buxton A, Gold S, Lange B, Woods W. Outcomes in childhood AML in the absence of transplantation in first remission—Children's Cancer Group (CCG) studies 2891 and CCG 213. Pediatr Blood Cancer. 2008;50:9-16.
14. Abrahamsson J, Clausen N, Gustafsson G, et al. Improved outcome after relapse in children with acute myeloid leukaemia. Brit J Haematol. 2007; 136:229-236.
15. Creutzig U, Zimmermann M, Ritter J, et al. Definition of a standard-risk group in children with AML. Brit J Haematol. 1999;104:630-639.

Downloaded from http://ashpublications.org/blood/article-pdf/111/5/2899/486868/zh800508002899.pdf by guest on 12 December 2024