High Expression Levels of X-Linked Inhibitor of Apoptosis Protein and Survivin Correlate with Poor Overall Survival in Childhood de Novo Acute Myeloid Leukemia

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ABSTRACT

Purpose: Apoptosis-related proteins are important molecules for predicting chemotherapy response and prognosis in adult acute myeloid leukemia (AML). However, data on the expression and prognostic impact of these molecules in childhood AML are rare.

Experimental Design: Using flow cytometry and Western blot analysis, we, therefore, investigated 45 leukemic cell samples from children with de novo AML enrolled and treated within the German AML-BFM93 study for the expression of apoptosis-regulating proteins [CD95, Bcl-2, Bax, Bcl-xL, procaspase-3, X-linked inhibitor of apoptosis protein (XIAP), cellular inhibitor of apoptosis protein–1 (cIAP-1), survivin].

Results: XIAP (P < 0.002) but no other apoptosis regulators showed maturation-dependent expression differences as determined by French-American-British (FAB) morphology with the highest expression levels observed within the immature M0/1 subtypes. XIAP (P < 0.01) and Bcl-xL (P < 0.01) expression was lower in patients with favorable rather than intermediate/poor cytogenetics. After a mean follow-up of 34 months, a shorter overall survival was associated with high expression levels of XIAP [30 (n = 10) versus 41 months (n = 34); P < 0.05] and survivin [27 (n = 10) versus 41 months (n = 34); P < 0.05].

Conclusions: We conclude that apoptosis-related molecules are associated with maturation stage, cytogenetic risk groups, and therapy outcome in childhood de novo AML. The observed association of XIAP with immature FAB types, intermediate/poor cytogenetics, and poor overall survival should be confirmed within prospective pediatric AML trials.

INTRODUCTION

During the last years, it became evident that apoptosis-related molecules may be useful as prognostic markers because several antiapoptotic mechanisms are operational in acute leukemias. Suppression of apoptosis contributes to leukemogenesis by different mechanisms, including prolonging cell life span, thus facilitating the accumulation of gene mutations, permitting growth factor-independent cell survival, promoting resistance to immune-based cytotoxicity, and allowing disobeysance of cell cycle checkpoints which would normally induce apoptosis. Defects in apoptotic mechanisms also play an important role in resistance to chemotherapy (1–3). In adult acute myeloid leukemia (AML), the expression patterns and prognostic role of CD95 (Fas/APO-1), molecules of the Bcl-2 and caspase families as well as members of the inhibitors of apoptosis proteins (IAPs) have been investigated in a variety of studies (4–15). In contrast, systematic data on the expression and prognostic impact of these molecules in pediatric AML are rare.

Childhood AML has a better treatment outcome than adult AML. For the current chemotherapy regimens, these prognostic differences are true for response to induction chemotherapy, relapse frequency, and overall survival (16–19). It has been speculated that differences in the maturation stage of the transformed progenitor cell clone contribute to the distinct prognosis of age-related AML groups. The leukemic cells in adult AML patients may arise from a transformed undifferentiated hematopoietic stem cell, whereas in childhood AML, the malignant cell clone potentially arises from a more committed myeloid progenitor cell. Different maturation stages of hematopoietic progenitor cells differ in their intrinsic resistance toward chemotherapy-induced cell death (i.e., immature stem cells are more resistant than committed myeloid progenitor cells), and this fact may contribute to the different treatment outcome of age-related AML groups (20, 21). Thus, age-related acute leukemia groups might be associated with distinct expression patterns of apoptosis-related molecules leading to the distinct prognosis in adult and childhood acute leukemia (11, 22, 23).

The main aims of this study were to analyze the expression patterns and prognostic relevance of apoptosis-related proteins...
in childhood de novo AML; to correlate expression with karyotype, maturation stage (FAB subtypes, immunophenotype), and outcome (response to induction chemotherapy, relapse frequency, overall survival) of pediatric AML cases who entered the AML-BFM93 study and, thus, received standardized diagnostic work-up and therapy, and to assess the applicability of these proteins as markers for treatment response.

**MATERIALS AND METHODS**

Patient Samples. Patient-derived cell samples (n = 45; bone marrow n = 35, peripheral blood n = 10) were consecutively collected, ficoll-purified, and cryopreserved between 1997 and 1999 within the German multicenter AML-BFM93 study. Diagnosis of AML was made morphologically based on FAB criteria (24, 25) and immunophenotypically according to European Group for the Immunological Characterization of Leukemias (EGIL) recommendations (26). All of the samples contained more than 80% leukemic cells based on morphological and immunophenotypical criteria. All of the patients were children and had de novo AML. Clinical characteristics of these patients are summarized in Table 1.

**Treatement According to German AML-BFM93 Study Protocol.** Treatment modalities and risk stratification of the AML-BFM93 study are described in detail elsewhere (18). In brief, during induction phase, patients either received either ADE (ara-C: 100 mg/m², day 1 to 8; daunorubicin: 30 mg/m² twice daily, days 3 to 5; etoposide: 150 mg/m², day 6 to 8) or AIE (idarubicin: 12 mg/m² daily instead of daunorubicin). Although AIE was associated with a better blast reduction in the bone marrow on day 15, long-term therapy outcome for both induction regimens was similar (27). After induction, patients were treated according to risk level (standard-risk group: FAB M1 or M2 with Auer rods, FAB M3, and FAB M4Eo with ≤5% blasts in the bone marrow on day 15; high-risk group: all others). Standard-risk patients were shifted to the high-risk group if they had more than 5% blasts in the bone marrow on day 15. Patients with FAB M3 were always treated within standard-risk group, regardless of blast count on day 15. All of the patients received 6 weeks of standard consolidation treatment with seven drugs (thioguanine 40 mg/m², day 1 to 43; prednisolone 40 mg/m², day 1 to 28; vincristine 1.5 mg/m² and doxorubicin 30 mg/m², day 1, 8, 15, an 22; ara-C 75 mg/m², day 3 to 6, 10 to 13, 17 to 20, 24 to 27, 31 to 34, and 38 to 41; intrathecal ara-C 40 mg in children <3 years, day 1, 15, 29, and 43; cyclophosphamide 500 mg/m², day 29 and 43). High-risk patients were randomized to receive additional consolidation treatment with HAM (high-dose ara-C 3 g/m² twice daily, days 1–3; mitoxantrone 10 mg/m², days 4 and 5) either before or after standard consolidation therapy. Randomized scheduling of HAM after induction did not reveal major differences in outcome. However, high-risk patients with ADE (ara-C, daunorubicin, etoposide) as induction therapy benefited especially from early HAM (18). After consolidation therapy, all of the patients were treated with an intensification block of high-dose ara-C and VP-16 (high-dose ara-C 3 g/m² twice daily for 3 days and etoposide 125 mg/m², day 2 to 5). This was followed by cranial irradiation with 18 Gy (standard dose in children ≥3 years) and maintenance therapy of thioguanine 40 mg/m² p.o. and ara-C 40 mg/m² s.c. for 4 days monthly for a total of 18 months.

Of the 45 patients in this series, 35 patients were treated according to high-risk therapy regimen and 10 patients received standard-risk treatment. Three patients died during induction chemotherapy, 3 patients were nonresponders, and 39 patients achieved complete remission (CR). Of the 39 patients with CR, 27 patients are in continuous CR (mean event-free survival: 42 months) and 12 patients suffered from relapse (mean event-free survival: 10 months). The mean overall survival follow up is 34 months.

**Expression Analysis of Apoptosis-Related Molecules CD95, Bel-2, and Bax by Flow Cytometry.** Surface CD95 expression and intracellular expression of Bcl-2 and Bax were determined as described previously using the phycoerythrin-conjugated anti-CD95 monoclonal antibody DX2, the FITC-conjugated anti-Bcl-2 monoclonal antibody 124, and the PE-conjugated anti-Bax monoclonal antibody I-19 raised against Bax-specific peptide sequences (11). Nonspecific binding due to Fc-receptors was blocked by preincubation of the cells with polyclonal rabbit serum (Life Technologies, Inc., Paisley, United Kingdom). Antigen expression distribution in individual cell samples was quantified as relative fluorescence intensity, determined by the ratio of mean fluorescence intensity of cells stained for the respective antigen to mean fluorescence intensity of the corresponding negative control. Bax:Bcl-2 ratio was determined by dividing the Bax relative fluorescence intensity value by the Bcl-2 relative fluorescence intensity value.

**Western Blot Analysis of Bel-xL, Procaspsase-3, XIAP, cIAP-1, and Survivin.** Immunoblotting was carried out using cell lysates from mononuclear cell fractions generated by ficoll separation. Detergent-lysates were prepared in the presence of protease inhibitors as described previously (8, 28). After normalization for total protein content (20 μg/Lane), samples were subjected to SDS-PAGE/immunoblot analysis using monoclonal antibodies specific for X-linked inhibitor of apoptosis protein (XIAP; Transduction Laboratories, Lexington, KY), cel-
Table 2 Correlation of expression of apoptosis-related proteins with French-American-British (FAB) morphology (M0/1, M2/3, M4/5) in childhood de novo acute myeloid leukemia

<table>
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<th>Expression levels</th>
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<th>Bcl-2</th>
<th>Bcl-xL</th>
<th>Procaspe-3</th>
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a n, number of examined samples; ns, not significant.

RESULTS

Constitutive Expression of Apoptosis-Related Proteins in Childhood de Novo AML: Correlation with FAB Morphology and Immunophenotype. Morphological FAB data were grouped into an immature subgroup (M0/1) and subtypes with granulocytic (M2/3) and myelomonocytic (M4/5) differentiation (Table 2). Immunophenotypic data were divided into immature phenotype (CD34, CD117 positivity) and phenotypic features of granulomonocytic (CD4, CD14, CD15, CD36, CD64, CD65, HLA-DR positivity) differentiation (Table 3).

Within this study, expression of CD95 was examined in 39 cell samples (Fig. 1B). In adult de novo AML, CD95 expression is higher in mature than in immature FAB subtypes (10, 11, 13). In contrast, expression of CD95 showed no clear maturation-dependent differences in this series of pediatric AML cases (Table 2). Expression of Bcl-2 was investigated in 38 cell samples (Fig. 1D). Bcl-2 expression is associated with immature FAB subtypes and CD34 expression in adult de novo AML (4, 11, 14, 15). Within this series of childhood AML samples, expression of Bcl-2 did not differ significantly between FAB subtypes and CD34 positive and negative cases (Tables 2 and 3).

XIAP expression levels were examined in 44 cell samples and were significantly higher in immature compared with more mature FAB subtypes (Table 2, Fig. 1A). For XIAP, this is in contrast to adult de novo AML; high expression of XIAP was associated with myelomonocytic FAB subtypes M4/5 and a monocytic-differentiated immunophenotype in a study of adult de novo AML patients (positivity for CD4, CD14, CD36, and HLA-DR) [5]. Similar to previously observed expression patterns in adult AML (11), expression of Bax (Fig. 1C), survivin, and cIAP-1 proteins (investigated in 38, 44, and 23 cell samples, respectively; Fig. 1A) showed no clear-cut correlations with

5 Unpublished data.
FAB subtypes (Table 2) and immunophenotype (data not shown) in this series of pediatric AML cases.

**Constitutive Expression of Apoptosis-Related Proteins in Childhood de Novo AML: Correlation with Cytogenetic Risk Groups.** Cytogenetic analyses were available for 37 patients and grouped into favorable and intermediate/poor risk categories as described in “Materials and Methods.” Interestingly, all of the cell samples of the seven patients with favorable cytogenetics were negative for XIAP and Bcl-xL. In contrast, XIAP and Bcl-xL were detectable in all of the cell samples of the 30 patients with intermediate/poor risk cytogenetics (XIAP: 259 ± 503 relative absorbance, P < 0.01; Bcl-xL: 2615 ± 439 relative absorbance, P < 0.01). Expression levels of none of the other analyzed proteins correlated with cytogenetic risk groups (data not shown).

**Constitutive Expression of Apoptosis-Related Proteins in Childhood de Novo AML: Correlation with Clinical Data and Therapy Response.** Procaspase-3 expression levels were positively correlated with age (r = 0.3, n = 44, P < 0.05) and WBC (r = 0.4, n = 44, P < 0.01). XIAP expression levels correlated with platelet count (r = 0.3, n = 44, P < 0.05). No additional significant correlations between apoptosis-related molecules and clinical data (age, WBC, platelet count, hemoglobin value, lactate dehydrogenase) were found. With the exception of XIAP (standard risk: mean relative absorbance = 780, n = 10; high risk: mean relative absorbance = 2948, n = 34; P < 0.05), expression levels of analyzed proteins did not differ significantly between patients treated according to high-risk therapy protocol and patients who received standard-risk treatment.

Three patients of the 45 examined patients were nonresponders to induction chemotherapy and 3 patients died during this therapy phase (“early deaths”). We observed no clear-cut expression differences for any of the analyzed proteins between nonresponders and patients with CR (n = 39) within this study (data not shown). Moreover, a significant expression difference between relapsed patients (n = 12) and patients in continuous CR (n = 27) was not seen for any of the investigated apoptosis-related molecules in this series (data not shown).

However, fitting with expectations based on the antiapoptotic activity of XIAP and survivin in cells, patients expressing high levels of XIAP (upper third expression level) had a shorter overall survival than patients expressing low levels of XIAP [mean, 30 (n = 10) versus 41 months (n = 34); P < 0.05; Fig. 2A]. When patient groups with XIAP expression levels above and below mean or median were compared, no significant differences in overall survival were observed for these groups (data not shown). Patients with detectable survivin protein expression had a shorter overall survival than patients negative for survivin [mean 27 (n = 10) versus 41 months (n = 34); P < 0.05; Fig. 2B]. In contrast, none of the other investigated proteins correlated with overall survival (data not shown). A recent study indicates a high Bax:Bcl-2 ratio as a favorable prognostic marker for response to induction therapy and overall survival in adult de novo AML (4). In contrast, in this study, children (n = 19) with a ratio above average had a shorter overall survival in
Fig. 1 Expression levels of apoptosis-related proteins in de novo acute myeloid leukemia (AML) cells. Examples of immunoblot (A) and flow cytometry (B–D) data are shown for three representative samples [French-American-British (FAB) morphology types M1, M3, M4]. A, immunoblotting was carried out using cell lysates from mononuclear cell fractions generated by ficoll separation. Detergent-lysates were prepared in the presence of protease inhibitors. After normalization for total protein content (20 μg/Lane), samples were subjected to SDS-PAGE/immunoblot analysis using monoclonal antibodies specific for X-linked inhibitor of apoptosis protein (XIAP), cellular inhibitor of apoptosis protein–1 (cIAP-1), survivin, procaspase-3, and Bcl-xL. Data on X-ray films were quantified by scanning-densitometry using the NIH Image analysis system. To normalize for variation in antibody concentration or time of exposure, the protein signal from the respective patient was normalized against the protein signal of the control cell line BJAB (B, human Burkitt-like lymphoma cell line). Expression levels are expressed in terms of this ratio (relative absorbance). Surface CD95 expression (B) and intracellular expression of Bax (C) and Bcl-2 (D) were determined using the phycoerythrin-conjugated anti-CD95 monoclonal antibody DX2, the FITC-conjugated anti-Bcl-2 monoclonal antibody 124, and the polyclonal rabbit-antihuman antibody I-19 raised against Bax-specific peptide sequences. Nonspecific binding due to Fc receptors was blocked by preincubation of the cells with polyclonal rabbit serum (Life Technologies, Inc.). Antigen expression distribution in individual cell samples was quantified as relative fluorescence (FL) intensity (RFI), determined by the ratio of mean fluorescence intensity of cells stained for the respective antigen to mean fluorescence intensity of the corresponding negative control.
this series compared with children \((n = 19)\) with a ratio below average \((33 \text{ versus } 40 \text{ months}; P = 0.175)\).

**DISCUSSION**

To determine the expression pattern and prognostic significance of apoptosis-related molecules in childhood \emph{de novo} AML, we investigated 45 cell samples of children enrolled and treated within the German AML-BFM93 trial.

Thus far, it is unclear why childhood AML has a better treatment outcome compared with adult AML. Differences in the maturation stage of the transformed progenitor cell clone, including distinct expression patterns of apoptosis-related molecules, might contribute to the distinct prognosis of age-related AML groups \((20, 21, 23)\). Previously, we showed that the overall expression levels of certain apoptosis-related molecules (cIAP-1, Bax, procaspase-3, XIAP) differ between childhood and adult AML \((22)\). In this study, we provide further evidence that expression patterns of pro- and antiapoptotic molecules vary between these age groups. The previously described association of CD95 \((10, 11, 13)\) and Bcl-2 \((4, 14, 15, 22)\) expression with certain maturation stages of the leukemic cells in adult AML was not seen in this pediatric AML series. This finding indicates that gene transcription and protein expression of these molecules may be differently regulated within distinct age-related AML groups.

Comparable with our previous findings in adult AML and normal peripheral blood cells,\(^{5}\) XIAP expression was higher in myelomonocytic-differentiated AML cells than in AML cells with granulocytic differentiation. Thus, the results of this study provide further evidence that XIAP plays a role in malignant as well as in normal monocytic differentiation. This assumption is also supported by the finding that XIAP is linked to monocytic differentiation in bryostatin1-treated leukemia cell lines \emph{in vitro} and that down-regulation of XIAP blocks monocytic differentiation \((31)\). Moreover, a recent study showed that induction of monocytic differentiation in the HL-60 cell line model leads to a marked up-regulation of XIAP, whereas during granulocytic differentiation, XIAP levels progressively declined \((32)\). It will be interesting to study the molecular mechanisms for the differential expression of IAPs in AML cells in more detail. For example, recent data suggest that XIAP may be regulated by the phosphatidylinositol 3-kinase pathway in AML \((33)\).

Cytogenetics are currently the most clearly defined prognostic factors in AML. Recent studies revealed important molecular insights in the transformational potential of specific leukemia-associated chromosomal abnormalities \((34)\). Still, it is mostly unclear why genetic subgroups differ in their chemotherapeutic response and whether this is related to differences in cellular drug resistance \((35)\). Fitting with the antiapoptotic activity of XIAP and Bcl-xL, the observed lower expression of these molecules in the favorable cytogenetic risk group might contribute to the better prognosis of this risk group. This significant lower XIAP expression within the favorable cytogenetic risk group was also observed in our previous study in adult \emph{de novo} AML.\(^{5}\)

Despite great advances in supportive care, chemotherapy is still associated with a significant amount of toxicity. The AML-BFM group currently investigates whether standard-risk patients in addition to the high-risk group might also benefit from an intensified consolidation therapy with HAM or whether this approach will lead to a significant increase of toxic side-effects outweighing the anticipated survival benefit \((18)\). Thus, additional potential prognostic markers to define subgroups that might benefit from specifically tailored therapy approaches are urgently needed. The newly described association between high expression levels of the antiapoptotic proteins XIAP and survivin and a shorter overall survival in this series defines these molecules as potential prognostic markers in childhood AML. For XIAP, this is in line with our previous finding suggesting this molecule as a potential prognostic marker in adult AML \((8)\).
In contrast, we could not determine a prognostic role for survivin in adult AML. Survivin is highly expressed and is cytokine regulated in myeloid leukemias and is a negative prognostic marker in a variety of solid tumors and diffuse large B-cell lymphomas (1, 36). For example, it has been reported that survivin expression in neuroblastomas correlates with clinically more aggressive, histologically unfavorable disease (37). Higher levels of survivin protein and p53 accumulation (indicative of mutant p53) were positively correlated in a survey of gastric cancers, implying an association of survivin with more aggressive disease (38). Adida et al. (9) found no significant difference in remission rate or survival in adult AML patients expressing high versus low levels of survivin. However, survivin expression became an independent negative prognostic factor for survival when adjusted for established prognostic factors (cytogenetics, age, and WBC).

At least two limitations of this study must be kept in mind for any further interpretation of the obtained results: (a) cryopreserved cell samples were used for our investigations. Therefore, this series might include a selection bias toward samples from patients with high leukocyte counts. The observed average leukocyte count within this series is indeed about 3-fold higher compared with the reported one for the overall ALL-BFM93 study (18). In addition, cryopreservation may alter protein expression in samples. Freezing and DMSO are proapoptotic stimuli potentially influencing expression levels of the proteins measured; (b) as summarized in the “Materials and Methods” section and explained in detail in the original publication (18), the AML-BFM93 treatment protocol included randomization steps and risk stratification. Thus, the number of investigated patients in this series might not be sufficient to draw definitive conclusions on the prognostic impact of the investigated apoptosis-related proteins. However, the observed impact of XIAP and survivin expression for overall survival within this series should lead to the prospective evaluation of these molecules within large pediatric AML therapy studies.

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