Drug resistance in childhood acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) is associated with impaired ability to induce apoptosis. To elucidate causes of apoptotic defects, we studied the protein expression of Apaf-1, procaspases-2, -3, -6, -7, -8, -10, and poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP) in cells from children with acute lymphoblastic leukemia (ALL; n = 43) and acute myeloid leukemia (AML; n = 10). PARP expression was present in all B-lineage samples, but absent in 4 of 15 T-lineage ALL samples and 3 of 10 AML cases, which was not caused by genomic deletions. PARP expression was a median 7-fold lower in T-lineage ALL (P < .001) and 10-fold lower in AML (P < .001) compared with B-lineage ALL. PARP expression was 4-fold lower in prednisolone, vincristine and L-asparaginase (PVA)–resistant compared with PVA-sensitive ALL patients (P < .001). Procaspase-2 expression was 3-fold lower in T-lineage ALL (P = .022) and AML (P = .014) compared with B-lineage ALL. In addition, procaspase-2 expression was 2-fold lower in PVA-resistant compared to PVA-sensitive ALL patients (P = .042). No relation between apoptotic protease-activating factor 1 (Apaf-1), procaspases-3, -6, -7, -8, -10, and drug resistance was found. In conclusion, low baseline expression of PARP and procaspase-2 is related to cellular drug resistance in childhood acute lymphoblastic leukemia. (Blood. 2005;106:1817-1823)
Patients, materials, and methods

Leukemia samples

Bone marrow and peripheral blood samples were obtained at initial diagnosis after informed consent from children with newly diagnosed ALL who were enrolled on protocol Dutch Childhood Oncology Group (DCOG) ALL-9 at the Erasmus MC/Sophia Children’s Hospital or on COALL-97 treatment protocol at one of the hospitals participating in the German COALL study group. Approval was obtained from the Erasmus MC/Sophia Children’s Hospital institutional review board for these studies. Mononuclear cells were isolated by sucrose density gradient centrifugation (Lymphoprep, density 1.077 g/mL; Nycomed Pharma, Oslo, Norway), within 24 hours after sampling at the research laboratory of Pediatric Oncology, Erasmus MC/Sophia Children’s Hospital, Rotterdam, The Netherlands. Cells were resuspended in culture medium consisting of RPMI 1640 (Dutch modification without l-glutamine; Gibco BRL, Life Technologies, Breda, The Netherlands) supplemented with 20% fetal calf serum (FCS; Integro, Zaandam, the Netherlands), 2 mM l-glutamine, 200 μg/mL gentamycin (Gibco BRL) 100 IU/mL penicillin, 100 μg/mL streptomycin, 0.125 μg/mL fungizone (Gibco BRL), and 5 μg/mL insulin, 5 μg/mL transferring, and 5 ng/mL sodium selenite (bovine insulin, human transferrin, and sodium selenite [ITS] media supplement; Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands). If necessary, leukemic samples were further enriched to at least 85% leukemic blasts by removing nonmalignant cells with immunomagnetic beads (Dynabeads, Dynal Oslo, Norway).23 Cell pellets were immediately stored at −80°C until use.

Antibodies

Monoclonal mouse antibodies to human procaspase-2 or PARP were purchased from PharMingen (San Diego, CA), Polyclonal rabbit antibodies to human procaspase-6, procaspase-10, and a monoclonal mouse antibody to human procaspase-8 were purchased from Cell Signaling Technology (Beverly, MA). In addition, we used monoclonal mouse antibodies to human procaspase-3 (Transduction Laboratories, Lexington, KY) and procaspase-7 (StressGen, Victoria, BC, Canada). Monoclonal antibodies tested for human procaspase-9 were purchased from Oncogene Research Products (Cambridge, MA), Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology.

In vitro drug resistance assay

Responsiveness of leukemia cells to PRED, VCR, ASP, and DNR was determined by the 4-day in vitro (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) MTT drug resistance assay as described previously.14 The concentration ranges tested for these drugs were as follows: PRED, 0.008-250 μg/mL; VCR, 0.05-50 μg/mL; ASP, 0.003-10 IU/mL; and DNR, 0.002-2.0 μg/mL. The drug concentration lethal to 50% of the ALL cells (LC50 value) was used as the measure of cellular drug resistance. The PRED, VCR, and ASP (PVA) score was calculated as individual scores for PRED, VCR, and ASP resulted in the PVA score. Patients with a combined PVA score of 3 or 4 are relatively sensitive to PRED, VCR, and ASP, and have a favorable treatment outcome compared with patients with a combined PVA score of 7, 8, or 9, which are relatively resistant to these 3 drugs.

Western blotting

Leukemic cells were lysed in 50 mM Tris (tris(hydroxymethyl)aminomethane; pH 7.6) containing 150 mM NaCl, 10 mM EDTA (ethylene-diaminetetraacetic acid), and 1% Triton X-100. Protease inhibitors (200 μg/mL pepstatin A, 200 μg/mL leupeptin and 2 mM PMSF [phenylmethylsulfonyl fluoride]) were freshly added prior to use. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). All samples were kept on ice during the protein isolation and lysates were either used directly or stored immediately at −80°C until use. Twenty micrograms of protein in Laemmli buffer (4% [wt/vol] sodium dodecyl sulfate [SDS], 100 mM Tris-HCl [pH 6.8], and 20% glycerol) was heated to 100°C for 3 minutes and loaded onto a 5% to 15% gradient polyacrylamide gel. After electrophoresis, samples were transferred to nitrocellulose blots, blocked with skim milk, and incubated for 1 hour with primary antibodies. Blots were washed and incubated for 1 hour with peroxidase-conjugated secondary antibody (DAKO, Carpientaria, CA). Presence of proteins was detected by enhanced chemiluminescent staining using the SuperSignal West Femto kit (Pierce). Signals were directly scanned by the ChemiGenius imaging system (SynGene, Cambridge, United Kingdom) and signal intensity was quantified using GeneTools 3.1 image analysis software (SynGene).

Quantification of apoptotic proteins

A dilution series of 30, 25, 20, 15, and 10 μg protein lysate of the human B-cell leukemia cell line Reh (N CRL-8286; ATCC, Rockville, MD) was included in each gel. Expression levels of protein were expressed in arbitrary units (AU) and were based on the relative quantity present in the patient’s sample compared to the Reh cell line dilution series (Figure 1). Reproducibility of our method was assessed by subjecting 27 samples to duplicate Western blot analysis and is illustrated in Figure 2 for 16 representative samples.
Real time quantitative PCR

Total cellular mRNA was extracted using Trizol reagent (Gibco BRL) and cDNA was synthesized using random hexamers and oligo dT primers. mRNA expression levels of PARP and GAPDH as a reference, were quantified using real-time polymerase chain reaction (PCR) analysis on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) as previously described.23 PCR reactions were performed and optimized to an amplification efficiency of more than 95%. The comparative cycle time (Ct) value of the target PCR was normalized by subtracting the Ct value of GAPDH (ΔCt). The ΔCt value was used to calculate the relative expression level to GAPDH for each target PCR using the following formula: relative mRNA expression = 2^(-ΔCt) × 100%.25 PARP primer sequences used were: sense, 5'-AGGCTGCTTTGTCAAGAA-3' and antisense, 5'-CTTGCTGCTTGTTGAAGAT-3' and the probe sequence: 5'-ATGAGGTTGATGGATGTA-3'. GAPDH primers and probe sequences have been described elsewhere.26

Fluorescence in situ hybridization analysis

To investigate the PARP gene copy number, dual colored fluorescence in situ hybridization (FISH) was applied using 2 combinations of probes: (1) Two Iq42-specific bacterial artificial chromosome (BAC) clones (Roswell Park Cancer Institute, Buffalo, NY), RP11-118H4 labeled with biotin-16-deoxyuridine triphosphate (dUTP), and RP11-125A15 labeled with digoxigenin-12-dUTP, with a 50-kilobase (kb) overlap in the PARP gene; and (2) a combination of the PARP-specific BAC clone RP11-118H4 (labeled with biotin-16-dUTP) and 2 P1-derived artificial chromosome (PAC) clones 203H23 and 213H16 (RCPI-6 Human PAC Library, Roswell Park Cancer Institute) labeled with digoxigenin-12-dUTP specific for the AF1q gene on Iq1. Cytosplms of leukemic samples stored at −20°C were used for FISH analysis. The FISH protocol was based on that described previously.27 Briefly, slides were pretreated with RNase and pepsin and subsequently fixed with formaldehyde. Hybridization of 100 ng of each probe to the slides was performed overnight at 37°C. Biotinylated probes were detected via subsequent incubation with fluorescein isothiocyanate (FITC)–labeled avidin-d, biotinylated goat–anti-avidin and avidin-d FITC (Vector, Burlingame, CA), whereas digoxigenin-labeled probes were detected via sheep–anti-digoxigenin tetramethylrhodamine isothiocyanate (TRITC)–labeled antibodies (Boehringer Mannheim, Mannheim, Germany), followed by Texas-red–conjugated donkey antiovidin antibodies (Jackson ImmunoResearch, Westgrove, PA). Slides were counterstained with DAPI (4',6-diamidino-2-phenylindole) and embedded in Vectashield (Vector Laboratories, Burlingame, CA)/DABCO (1,4-diazabicyclo[2,2,2]octane). Hybridization results were examined using a Zeiss Axioplan 2 fluorescence microscope (Zeiss, Oberkochen, Germany).

Statistics

Protein expression in different leukemic subgroups (B-lineage ALL, T-lineage ALL, and AML) was compared using the Mann-Whitney U test. Spearman rank (r_s) correlation test was used to correlate protein expression to several variables on the study. Statistical tests were performed at a 2-tailed significance level of .05. Whenever applicable, a Bonferroni correction was applied to allow for multiple comparisons.

Results

Expression of various apoptotic proteins in ALL

The expression of Apaf-1, procaspases-2, -3, -6, -7, -8, -10, and PARP was first determined in a test group of 20 children with ALL. These 20 patients were selected by having a PVA score of 3 or 4 (ie, relatively in vitro–sensitive to PRED, VCR, and ASP; n = 10) or 7, 8, or 9 (ie, relatively in vitro–resistant to PRED, VCR, and ASP; n = 10). The expression of procaspase-9 could not be analyzed in these samples since all procaspase-9 antibodies tested revealed abundant nonspecific staining, whereas staining of procaspase-9 itself was low. Most other apoptotic proteins were ubiquitously expressed in the ALL samples and varied markedly between patients (Figure 3). Exceptions were procaspase-10 and PARP, which were not expressed in 4 (2 B-lineage ALL, 2 T-lineage ALL) and 1 (T-lineage ALL) out of the tested 20 samples, respectively. Expression values in arbitrary units (AU) for each apoptotic protein are provided in Table 2. The variation in expression ranged

<table>
<thead>
<tr>
<th>Apoptotic protein</th>
<th>PVA-sensitive patients, median AU (range)</th>
<th>PVA-resistant patients, median AU (range)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apaf-1</td>
<td>21.6 (12.7-124.4)</td>
<td>17.4 (4.0-175.3)</td>
<td>.529</td>
</tr>
<tr>
<td>Procaspase-2</td>
<td>13.4 (1.1-27.2)</td>
<td>6.2 (1.2-10.1)</td>
<td>.019</td>
</tr>
<tr>
<td>Procaspase-3</td>
<td>18.5 (9.9-41.0)</td>
<td>20.8 (8.0-35.7)</td>
<td>.912</td>
</tr>
<tr>
<td>Procaspase-6</td>
<td>17.5 (11.0-78.8)</td>
<td>25.8 (16.3-49.0)</td>
<td>.208</td>
</tr>
<tr>
<td>Procaspase-7</td>
<td>10.8 (6.1-32.4)</td>
<td>8.6 (1.0-25.2)</td>
<td>.247</td>
</tr>
<tr>
<td>Procaspase-8</td>
<td>32.4 (21.5-75.2)</td>
<td>42.3 (17.0-110.1)</td>
<td>.393</td>
</tr>
<tr>
<td>Procaspase-10</td>
<td>5.4 (0.0-40.8)</td>
<td>6.4 (0.0-47.0)</td>
<td>.912</td>
</tr>
<tr>
<td>PARP</td>
<td>35.8 (6.5-46.9)</td>
<td>15.1 (0.0-38.5)</td>
<td>.042</td>
</tr>
</tbody>
</table>

Expression of 8 apoptotic proteins was compared in 10 PVA-sensitive (8 B-lineage ALL, 2 T-lineage ALL) and 10 PVA-resistant (5 B-lineage, 5 T-lineage) patients. For both groups, n = 10.

The expression of the predominantly expressed isoform was examined for all proteins.

Expression of an apoptotic protein in patient samples related to the expression of protein in a dilution series of the Reh leukemic cell line; see “Materials and methods.”
between 5-fold for procaspase-3 and 44-fold for Apaf-1. In vitro cross-resistance to PVA is an important independent predictor of treatment failure and long-term outcome in childhood ALL.\(^{1,5,28}\)

Therefore, the expression of the 8 apoptotic proteins was subsequently correlated to in vitro PVA resistance. Only procaspase-2 and PARP protein expression were significantly associated with in vitro PVA resistance in ALL. Procaspase-2 expression was 2.2-fold lower in cells of ALL patients resistant to PRED, VCR, and ASP, hereafter called PVA resistant (median: 6.2 AU) compared with cells of ALL patients sensitive to these 3 drugs, hereafter called PVA sensitive (median, 13.4 AU; \(P = .019\)). PARP expression was 2.4-fold lower in PVA-resistant (median, 15.1 AU) compared with PVA-sensitive children with ALL (median, 35.8 AU; \(P = .042\)). Based on these data, we examined the expression of PARP and procaspase-2 in a larger group of children with ALL (\(n = 43\)) and AML (\(n = 10\)).

**Expression of PARP in acute leukemia**

Twenty-eight patients with B-lineage ALL, 15 patients with T-lineage ALL, and 10 patients with AML were studied. PARP expression varied between 0 and 59.8 AU in these patients with the relatively highest expression level found in B-lineage ALL (Figure 4A). The expression of PARP was significantly lower in T-lineage ALL (median, 4.3 AU; \(P < .001\)) and in AML (median, 3.1 AU; \(P < .001\)) compared to B-lineage ALL (median, 32.0 AU). PARP expression did not differ significantly between AML and T-lineage ALL. Whereas all patients with B-lineage ALL showed PARP expression, we identified 4 T-ALL and 3 AML patients without detectable PARP expression. PARP mRNA levels correlated with PARP protein levels (\(r_s = .58\), \(P = .018\)). FISH analysis of the 4 patients with T-lineage ALL and 3 patients with AML with absent PARP protein expression revealed that the lack of PARP protein and mRNA expression was not associated with genomic deletions in the **PARP** gene region of these patients (data not shown).

Epigenetic changes such as DNA methylation of promoter regions may result in decreased mRNA levels; however, no cytosine-phosphate-guanine (CpG) islands have been reported within the promoter region of **PARP**, nor could we identify these using the CpGPlot program (http://www.ebi.ac.uk/emboss/cpgplot/).\(^29\)

Table 3. PARP and procaspase-2 expression in childhood acute leukemia patients sensitive and resistant to PRED, VCR, ASP, and DNR

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. studied</th>
<th>Median, AU</th>
<th>(P)</th>
<th>No. studied</th>
<th>Median, AU</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRED sensitive</td>
<td>20</td>
<td>39.8</td>
<td>(&lt;.001^*)</td>
<td>15</td>
<td>24.7</td>
<td>(.012^*)</td>
</tr>
<tr>
<td>PRED resistant</td>
<td>21</td>
<td>6.9</td>
<td></td>
<td>19</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td>VCR sensitive</td>
<td>24</td>
<td>31.0</td>
<td>(.002^*)</td>
<td>19</td>
<td>22.0</td>
<td>(.142)</td>
</tr>
<tr>
<td>VCR resistant</td>
<td>21</td>
<td>4.7</td>
<td></td>
<td>18</td>
<td>9.3</td>
<td></td>
</tr>
<tr>
<td>ASP sensitive</td>
<td>21</td>
<td>31.7</td>
<td>(.004^*)</td>
<td>18</td>
<td>23.3</td>
<td>(.070)</td>
</tr>
<tr>
<td>ASP resistant</td>
<td>22</td>
<td>7.0</td>
<td></td>
<td>19</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>DNR sensitive</td>
<td>30</td>
<td>31.0</td>
<td>(.017)</td>
<td>24</td>
<td>12.8</td>
<td>(.188)</td>
</tr>
<tr>
<td>DNR resistant</td>
<td>9</td>
<td>4.7</td>
<td></td>
<td>8</td>
<td>7.3</td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant after Bonferroni correction for multiple comparisons.
expression of procaspase-2 was 3-fold lower in T-lineage ALL (median, 7.0 AU; \( P = 0.014 \)) and in AML (median, 6.80; \( P = 0.022 \)) compared with B-lineage ALL (median, 22.0 AU). Procaspase-2 expression did not differ significantly between AML and T-lineage ALL (\( P = 0.976 \)).

Procaspase-2 expression was compared between in vitro-sensitive and -resistant patients for PRED, VCR, ASP, or DNR as single drugs. After correction for multiple comparisons, we observed that decreased procaspase-2 expression was related to in vitro PRED resistance only, although a trend with resistance to the other drugs was found (Table 3). Within the total group of ALL patients, PVA-resistant cases (median, 9.7 AU) had a 2-fold lower expression of procaspase-2 compared with sensitive cases (median, 24.0 AU; \( P = 0.042 \); Figure 5B). Within the B-lineage group, PVA-resistant patients (n = 6; median, 11.8 AU) had a 2.1-fold lower expression of procaspase-2 compared with sensitive patients, although this difference was not statistically significant (n = 15, median, 24.7 AU, \( P = 0.132 \); Figure 5B, open symbols).

Since the expression of procaspase-2 and PARP showed similar expression patterns, we examined the possibility whether the expression of both proteins was correlated. As shown in Figure 6 there is a strong positive correlation between the expression of PARP and procaspase-2 (\( r_t = 0.58, P < 0.001 \)). Procaspase-2 expression did not differ between ALL patients with an M1 bone marrow response at day 15 and those having an M2 or M3 bone marrow response (\( P = 0.30 \)). Due to limited follow-up of patients no relevant comparisons with long-term outcome could be made.

**Discussion**

The prognosis of childhood acute lymphoblastic leukemia (ALL) has improved remarkably over the past 4 decades due to the introduction of effective combination risk-adapted therapies. Conventional factors used to stratify patients are clinical and biological parameters such as age and white blood cell (WBC) count at diagnosis, immunophenotype, the presence of specific genetic abnormalities, and initial response to PRED treatment. Newer approaches are measurement of minimal residual disease after induction of initial remission and in vitro drug resistance profiles. We and others showed that children whose leukemic cells exhibit in vitro resistance to antileukemic agents, especially to PRED, VCR, and ASP, have a significantly worse prognosis than patients whose ALL cells are sensitive. Children with T-lineage ALL and AML are, in vitro, more resistant to a variety of drugs. The use of more intensive risk-adapted treatment regimens, however, has resulted in a prognosis for children with T-lineage ALL similar to that of children with B-lineage ALL. In the present study, the expression of Apaf-1, procaspases-2, -3, -6, -7, -8, -10, and PARP was studied in childhood ALL and AML and was related to immunophenotype and in vitro resistance to PRED, VCR, and ASP (PVA) as well as to resistance to PRED, VCR, and ASP and DNR individually.

We observed no relation between the expression of Apaf-1, procaspases-3, -6, -7, -8, and -10, and PVA resistance in childhood ALL in the present study. The absence of a relation with drug resistance may explain why no prognostic value was found for these proteins in adult ALL and AML, although data in children and adults are not necessarily interchangeable. In a previous study we demonstrated that the degree of caspase-3 activation (ie, cleavage of procaspase-3 into caspase-3) correlates with the degree of resistance to PRED and ASP in childhood ALL. In our present study no correlation was found between the baseline expression levels of procaspase-3 and resistance to these chemotherapeutic drugs. Taken together, these studies suggest that resistance to these 2 drugs may not be caused by decreased availability of the active enzyme due to decreased expression of procaspase-3, but rather may be caused by defects upstream of caspase-3 that inhibit caspase-3 activation upon PRED or ASP exposure.

PARP (Enzyme Commission [EC] 2.4.2.30) is an abundantly expressed nuclear enzyme, which binds to single- or double-stranded DNA breaks in response to DNA damage. At the breakage site, PARP catalyzes the transfer of the ADP-ribose polymers from the respiratory coenzyme NAD⁺ to nuclear acceptor proteins involved in chromatin structure, DNA repair, and DNA metabolism. Its poly(ADP-ribosylation) activity as well as its association with components of base-excision repair contribute to the role of PARP in DNA repair and the maintenance of genomic integrity. In the present study, we observed that in vitro drug-resistant childhood leukemia subtypes (ie, T-lineage ALL and AML), have a decreased and occasionally even absent expression of PARP. Complete absence of PARP expression was observed in 4 out of 15 patients with T-lineage ALL and 3 out of 10 patients with AML, but in none of the patients with B-lineage ALL. The variation in PARP expression between leukemia subtypes suggests that caution should be taken when interpreting studies that examining protein expression in a mixed group of B-lineage ALL, T-lineage ALL, and AML samples without correcting for leukemia subtypes. The absence of PARP was not caused by deletions of the PARP gene. Given the absence of CpG islands within the PARP promoter, it seems
unlikely that the absence of PARP is caused by methylation-induced PARP silencing in these patients. The presence of putative binding sites for several transcription factors, including simian virus 40 protein 1 (Sp-1), activator protein-2 (AP-2), 4-v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets-1), and yin-yang 1 (YY1)9 within the PARP promoter region, suggests that PARP expression is regulated at the transcriptional level. In addition, we observed that decreased or absent expression of intact PARP is related to in vitro resistance to PRED, VCR, and ASP in childhood ALL. Other studies also reported that depletion of PARP, either by gene disruption,40 antisense RNA,41 or pharmacologic inhibitors,40,43-45 resulted in decreased drug-induced apoptosis. Various explanations have been proposed for the requirement of PARP during apoptosis, including depletion of cellular nicotinamide-adenine nucleotide (NAD)⁺ and adenosine triphosphate (ATP) pools, modification of proteins involved in apoptosis like p53,47,48 facilitation of oligonucleosomal DNA fragmentation,49 and up-regulation of P-glycoprotein in PARP-depleted cells.41 We previously observed that resistance to PRED and ASP, but not to VCR and DNR, is linked to decreased drug-induced PARP cleavage (ie, inactivation of PARP).17 Our current observation that resistant cells express lower baseline levels of this protein suggests that the suitability to use cleaved PARP as read-out for functional apoptosis is drug/stimuli dependent.

Caspase-2 activity is implicated in the initiation50,51 as well as the effector phase52,53 of apoptosis induced by various stimuli. The present study shows a decreased procaspase-2 expression in vitro drug-resistant childhood leukemia subtypes (ie, T-ALL and AML). In addition, we observed a relation between decreased procaspase-2 expression and in vitro PVA resistance in childhood ALL. This suggests that sufficient intracellular amounts of procaspase-2 are required to respond to these drugs, and that relative deficits in procaspase-2 expression levels may contribute to cellular PVA resistance in childhood acute leukemia. In adult acute leukemia, high expression of procaspase-2 was not linked to prognosis.19-22 The lack of prognostic value for the response to combination chemotherapy in these studies does not rule out a role for procaspase-2 in cellular responses to PRED and/or other drugs in adult leukemias. Since this relationship has not been addressed in adults so far, no comparison between childhood and adult studies can be made. In cell lines the requirement of caspase-2 for drug-induced apoptosis was shown to be highly cell specific.54,55 Decreased PARP and procaspase-2 expression did correlate with in vitro PVA resistance, whereas these expression levels did not correlate with an early bone marrow response at day 15. One may argue that this is exactly the time period when the leukemia is treated with PRED, VCR, and ASP in most protocols. However, in the COALL-97 protocol, patients are first treated with a DNR window (up-front combination chemotherapy) for 7 days, followed by PRED, VCR, and DNR for 4 weeks. After this period of 5 weeks, ASP is given for the first time. Since PARP and procaspase-2 expression are not linked to DNR resistance, and this drug is used up-front of combination chemotherapy as well as in the first 4 weeks, this may explain the lack of correlation between both proteins and day-15 bone marrow response of COALL-97 patients.

In our previous study we observed that resistance to each of the 4 studied drugs (PRED, VCR, ASP, and DNR) was associated with a decreased ability to expose phosphatidyl serine on the outer membrane and decreased collapse of the mitochondrial membrane potential.17 Caspase-3 activation and PARP inactivation correlated to PRED and ASP resistance, but not to VCR and DNR resistance. This finding suggested that cleaved caspase-3 and PARP are not important determinants of the VCR- and DNR-triggered apoptotic route. Both phosphatidyl serine exposure and the collapse of the mitochondrial membrane potential occurred earlier than caspase-3 activation and PARP inactivation, implying that resistance to these 4 drugs is caused by a blockade at the level of or upstream of mitochondrial function. The identity of these upstream causes of resistance are part of extensive research in the past few years by different groups (reviewed by Pieters and Den Boer56).

Recently, new insights were obtained by comparing gene expression profiles of drug-resistant and -sensitive cells of children with ALL.24 In this latter study, drug-specific genes rather than more general apoptosis-associated genes (such as caspases, Bcl-2 family members, and inhibitors of apoptosis [IAP] factors) were among the most discriminative genes that were associated with resistance to PRED, VCR, ASP, and DNR in ALL cells. These findings do not rule out a role for altered expression and/or activity of apoptosis genes, but suggest that, at the mRNA level, genes upstream of the apoptosis-execution pathway are more important for resistance to these drugs. At the protein level, we here show that the basal expression levels of Apaf-1 and procaspase-3, -6, -7, -8, and -10 are not differentially expressed between drug-resistant and -sensitive patients with acute leukemia. Expression of procaspase-2 and PARP were decreased in children with drug-resistant ALL. The causal aspect of this association should be further determined with functional studies showing the sensitizing effect of ectopic expression of PARP and procaspase-2 on drug resistance in leukemic cells of children with ALL.

Acknowledgments

We thank all the hospitals participating in the German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL) and the staff of the Erasmus MC/Sophia Children's Hospital for providing the leukemia samples. In addition, we thank Renée X. Menezes (biostatistician) for statistical advice.

References