Up-regulation of asparagine synthetase expression is not linked to the clinical response to L-asparaginase in pediatric acute lymphoblastic leukemia


L-asparaginase (L-Asp) is an effective drug for treatment of children with acute lymphoblastic leukemia (ALL). The effectiveness is generally thought to result from a rapid depletion of asparagine in serum and cells. Asparagine synthetase (AS) opposes the action of L-Asp by resynthesis of asparagine. In vitro, resistance to L-Asp has been associated with up-regulation of AS mRNA expression. We monitored AS mRNA levels in leukemic cells before and during 5 days after intravenous administration of 1000 IU/m² pegylated L-asparaginase (PEG-Asp) in a therapeutic window in children with ALL at initial diagnosis. Within 24 hours, AS mRNA levels increased by 3.5-fold and remained stable in the following 4 days. Baseline and L-Asp–induced expression levels of AS did not differ between clinically good, intermediate, and poor responders to PEG-Asp. No significant difference of AS mRNA up-regulation was found between precursor B- and T-ALL or between hyperdiploids, TEL/AML1 rearranged ALL or absence of genetic abnormalities. In 3 of 12 patients with T-ALL even a slight down-regulation of AS mRNA expression upon L-Asp exposure was found. In conclusion, although L-Asp exposure induces the expression of AS mRNA, the up-regulated gene expression does not correlate with an early clinical poor response to this drug in children with ALL.

© 2006 by The American Society of Hematology

Patients, materials, and methods

Patients and therapeutic window with PEG-Asp

In close collaboration between our institution and the Dutch Childhood Oncology Group (DCOG; the former Dutch Childhood Leukemia Study Group), a window study with pegylated Escherichia coli L-Asp (PEG-Asp) upfront to the ALL-9 treatment schedule was initiated in July 2000. The DCOG ALL-9 study was implemented in the Netherlands to confirm the good results of the ALL-6 study,10 which was originally based on German ALL-BFM strategy. The aim of our study is to determine the clinical response as well as molecular determinants of L-Asp response in ALL. Children with ALL at initial diagnosis and presenting with white blood count (WBC) greater than 10 × 10⁹/L were eligible. Similar to a study from the Dana Farber Cancer Institute,11 we assessed a 5-day investigational window. A complete and persistent depletion of asparagine is considered to be the mechanism of action of L-Asp treatment. Boos et al12 showed that a plasma E coli AS activity of more than 100 IU/L leads to an asparagine depletion of less than 0.2 μM in plasma. Muller demonstrated that one dose of 1000 IU/m² PEG-Asp resulted in more than 100 U/L L-Asp activity for at least 10 days in children with ALL at initial diagnosis and expressing with white blood count (WBC) greater than 10 × 10⁹/L were eligible. Similar to a study from the Dana Farber Cancer Institute,11 we assessed a 5-day investigational window. A complete and persistent depletion of asparagine is considered to be the mechanism of action of L-Asp treatment. Boos et al12 showed that a plasma E coli AS activity of more than 100 IU/L leads to an asparagine depletion of less than 0.2 μM in plasma. Muller demonstrated that one dose of 1000 IU/m² PEG-Asp resulted in more than 100 U/L serum enzyme activity of L-Asp for 3 weeks.13 In a previous study we confirmed that 1000 IU/m² PEG-Asp given as a therapeutic window at day 5 (ie, 5 days before starting combined chemotherapy) results in more than 100 U/L L-Asp activity for at least 10 days in children with ALL at initial diagnosis.14 In the present study patients received a single dose of 1000 IU/m² PEG-Asp in a 1-hour infusion 5 days before starting the DCOG ALL-9 combination

From the Department of Pediatric Oncology/Hematology, Erasmus MC/Sophia Children’s Hospital, Erasmus University Medical Center, Rotterdam, The Netherlands; and the Dutch Childhood Oncology Group, The Hague, The Netherlands.


Reprints: Monique L. den Boer, Pediatric Oncology/Hematology, Erasmus MC/Sophia Children’s Hospital, Rotterdam, PO Box 2060, 3000 CB Rotterdam, The Netherlands; email: m.l.denboer@erasmusmc.nl.

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 U.S.C. section 1734.

© 2006 by The American Society of Hematology
Table 1. Characteristics of 31 patients treated with one dose of PEG-Asp before the DCOG-ALL9 study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/female</td>
<td>17/14</td>
</tr>
<tr>
<td>Age, median, y (range)</td>
<td>4.2 (1.2-13.1)</td>
</tr>
<tr>
<td>Median WBC count at diagnosis, × 10⁹/L (range)</td>
<td>47 (11.4-417)</td>
</tr>
</tbody>
</table>

**Immunophenotype**

- Pro-B-ALL: 1
- Common ALL: 9
- Pre-B-ALL: 9
- T-ALL: 12

**Cytogenetic characteristics**

- Hyperdiploid: 9
- TEL-AML1 fusion: 5
- BCR-ABL fusion: 5
- MLL gene rearranged: 1
- Others: 17

**CNS involvement†**

- Yes: 0
- No: 31

Table 2. Asparagine synthetase (AS) mRNA expression values in time

<table>
<thead>
<tr>
<th>Expression of AS compared with GAPDH, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day</td>
</tr>
<tr>
<td>Median</td>
</tr>
<tr>
<td>25th–75th percentile</td>
</tr>
</tbody>
</table>

**Wilcoxon signed rank test, P**

- Compared between successive days:
  - NA < .001
  - .4
  - .4
  - .06
  - .4

- Day – 5 values compared with levels of day – 4, – 3, – 2, – 1, and 0
  - NA < .001
  - .001
  - < .001
  - NA < .001
  - .001
  - .002

*Mean median expression values (25th–75th percentiles of AS mRNA compared with GAPDH in time in leukemic cells of 31 children induced by pegylated L-asparaginase (PEG-Asp). Wilcoxon signed rank test compared between successive days, and day – 5 values compared with levels of day – 4, day – 3, day – 2, day – 1, and day 0. NA indicates not applicable.*
Quantitative real-time PCR

The mRNA expression levels of AS and the endogenous housekeeping gene encoding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a reference, were quantified using real-time polymerase chain reaction (RTQ-PCR) analysis (TAQMAN chemistry) on an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster City, CA). Amplification of specific PCR products was detected using dual-fluorescent nontendextand probes labeled with 6-carboxyfluorescein (FAM) at the 5’ end and 6-carboxytetramethylrhodamine (TAMRA) at the 3’ end. Primers and probe combinations were designed using OLGIO 6.22 software (Molecular Biology Insights, Cascade, CO) and have been published elsewhere.18 Because all PCRs were performed with equal efficiencies (> 95%), relative mRNA expression levels of AS for each patient could be normalized for input RNA using the GAPDH expression of the patient. The relative mRNA expression level of the target gene in each patient was calculated using the comparative cycle time (Ct) method.19

Briefly, the target PCR Ct values (ie, the cycle number at which emitted fluorescence exceeds 10 x the standard deviation [SD] of baseline emissions, as measured from cycles 3 to 12) are normalized by subtracting the Ct value from the target PCR Ct value, which gives the ΔCt value. From the ΔCt value, the relative expression level to GAPDH for AS is calculated using the following equation: relative mRNA expression = 2^-ΔCt x 100%.

Statistics

Differences in mRNA expression levels measured at different days were analyzed using the Wilcoxon matched-pairs signed rank test. The relationship between AS mRNA expression and immunophenotype or cytogenetic subtype was analyzed with the Mann-Whitney U test.

Results

Children with newly diagnosed ALL and WBC greater than 10 x 10⁹/L were consecutively enrolled into the study. As is shown in Table 1, 31 children were eligible at the moment of analysis: 1 with pro–B-ALL, 9 with common ALL, 9 with pre–B-ALL, and 12 with T-ALL. Similar to day 7 for a prednisone window response, we evaluated the in vivo response to PEG-Asp by counting the number of leukemic blasts in the peripheral blood at day 0, 5 days after PEG-Asp was given. As can be seen in Figure 1, the leukemic cells in the peripheral blood dropped continuously over 5 days. The number of leukemic cells reduced 224-fold, from a median of 44.7 x 10⁹/L at day −5 to a median of 0.2 x 10⁹/L at day 0. This was more than a 2-log decrease in leukemic cell burden. There were 21 (68%) children who were PEG-Asp good responders (blast number < 1 x 10⁹/L at day 0), 6 (19%) who were intermediate responders (blasts 1 x 10⁹/L to 10 x 10⁹/L at day 0), and 4 (12%) who were poor responders (blasts > 10 x 10⁹/L at day 0) (Figure 1).

The baseline expression level of AS mRNA relative to GAPDH was a median of 0.26% (range, 0.05%-2.5%) in leukemic cells (> 90% purity). This was in the range of healthy controls as described in our previous study.16 The expression levels of AS in leukemic cells relative to GAPDH increased significantly a median of 3-fold, from 0.26% (basal expression) to 0.75% (24 hours later; P < .001; Figure 2). During the following 4 days the expression of AS mRNA remained stable at the level of 24 hours (Figure 2; Table 2).

The baseline expression level of AS mRNA did not differ between good and intermediate responders (P = .614), good and poor responders (P = .852), and intermediate and poor (P = 1.0) responders, nor did the up-regulated AS levels after 24 hours of PEG-Asp differ between good and intermediate (P = .614) responders, good and poor (P = .737) responders, and intermediate and poor (P = .914) responders (Figure 3A-B). The fold-change in AS mRNA expression levels was also not related to the relative (P = .997) or absolute (P = .804) decrease in leukemic cells in all 31 patients.

The AS expression for the different immunophenotypic ALL subgroups at diagnosis is shown in Figure 4. The median levels of AS mRNA relative to GAPDH mRNA for c/pre–B-ALL patients (0.21%) and for the T-ALL patients (0.28%) did not significantly differ (P = .376). One window patient had a pro–B-ALL for which the baseline expression of AS mRNA was 3-fold higher than the other c/pre–B-ALL patients. To explore whether pro–B-ALL is associated with a high AS mRNA expression we analyzed the AS expression of 23 infant pro–B-ALL cases. Infants with pro–B-ALL had a median 0.15% (range, 0.07%-1.43%) AS mRNA expression level, which was not significantly different from the baseline AS expression values in non-infants with c/pre–B- or T-ALL (Figure 4A). The c/pre–B-ALL group had a median baseline AS expression...
Figure 3. Relationship between asparaginase synthetase (AS) mRNA expression induced by pegylated L-asparaginase (PEG-Asp) and clinical response. (A) Baseline AS mRNA expression levels. (B) PEG-Asp-induced changes in AS mRNA expression levels measured after 24 hours of in vivo exposure to PEG-Asp compared with baseline expression levels. Dots represent individual expression values; solid lines, the median expression value per group. For definition of clinical response, see Figure 1.

level of 0.21%, which rose significantly to a median of 0.72% 1 day later (median 3.99-fold individual up-regulation, \( P < .001 \)). Patients with T-ALL demonstrated a significant increase from basal 0.28% to 0.68% 1 day later (median 1.94-fold individual up-regulation, \( P = 0.012 \)). Patients with T-ALL tended to have a lower individual up-regulation of AS mRNA compared with the children with \( \text{c/pre-B-ALL} \), but this was not statistically different (\( P = .107 \); Figure 4B). Only 3 cases had a slight down-regulation of the AS mRNA expression (−1.6, −1.2, and −1.05-fold). These 3 cases were all patients with T-ALL, of whom 2 were clinically good responders.

The baseline and Asp-induced expression levels of AS mRNA did not differ between hyperdiploid \( (n = 9) \), TEL/AML1–positive \( (n = 5) \), and other B-lineage ALL \( (n = 5) \). For infants with \( MLL \) gene–rearranged ALL, no data were available for the effect of L-Asp on AS mRNA levels, because these patients were not eligible for the PEG-Asp window study.

Discussion

Studies on putative causes of L-Asp resistance have been performed most extensively in mouse cell lines.5,6 L-Asp–sensitive tumor cells that did not contain detectable levels of AS developed resistance to L-Asp through exposure of cells to sublethal concentrations of the drug.20 Resistant cells up-regulated AS expression and activity by 60-fold. It is well known that AS plays a crucial role in maintaining amino acid homeostasis in cells.21 A rapid transcriptional control of the AS gene occurs following deprivation of any single essential amino acid.6,22 Hutson’s experiments were not comparable with in vivo situations, where various products and substrates (such as aspartate, glutamate, glutamine, and ammonia, among others) are all part of metabolic pathways and equilibrium conditions.5

In 1969 Haskell et al studied in vivo AS activity in 18 patients with leukemia.3 Prior to therapy, AS activity was nearly undetectable in leukemic cells. Patients were treated with 200 IU/kg \( E \) coli L-Asp daily for 3 days to 3 weeks. A 7-fold increase in AS activity was found in 5 L-Asp–resistant patients compared with 4 L-Asp–sensitive patients (mixed cohort of ALL, acute myeloid leukemia [AML], chronic myelogenous leukemia [CML], and chronic lymphocytic leukemia [CLL]). Haskell et al3 suggested that L-Asp resistance was related to the capacity of leukemic cells to up-regulate AS expression for asparagine biosynthesis. However, besides the limited number of patients in a very heterogeneous group, the criteria used to determine whether the patient was resistant or sensitive to L-Asp were not described.

In order to study the effect of monotherapy with L-Asp on leukemic blasts we used PEG-Asp. The effectivity of different L-Asp products like Erwinase, \( E \) coli ASP, or PEG-Asp is the same if the serum enzyme activity of L-Asp is higher than 100 U/L.13 We studied whether baseline levels or up-regulated levels of AS mRNA expression in leukemic cells after an in vivo treatment with PEG-Asp monotherapy were associated with short-term clinical response to this drug in children with ALL. The baseline AS expression levels were in the same range as healthy controls, as reported before.18 Up-regulation of AS mRNA occurred within 24 hours after PEG-Asp exposure and thereafter no further changes were found. Because the drop in leukemic cells was seen during the whole window period (Figures 1 and 2), it is unlikely that only leukemic cells resistant to PEG-Asp with intrinsic higher AS expression levels were left over on day −4. Baseline and L-Asp–induced AS mRNA expression levels did not differ between patients with good, intermediate, or poor response (Figure 3). So, L-Asp–induced up-regulation of AS mRNA is not related to early in vivo blast reduction in childhood ALL and thus is not predictive for the short-term clinical response to L-Asp. As mentioned earlier, cell
line studies showed that mRNA, protein, and activity levels of AS are correlated,6,7,22 but at present it is unknown whether this is also the case for clinical samples because only limited amounts of patients’ samples can be obtained. Immunophenotypic and genetic abnormalities are related to drug resistance and outcome in childhood ALL.25–27 T-ALL cells from children are, in vitro, more resistant to L-Asp than are cells from children with precursor B-lineage ALL.28 The relative resistance to L-Asp of T-ALL cases cannot be explained by altered expression of the AS gene, since both baseline and L-Asp–induced changes in AS mRNA expression did not differ between patients with T- and c/pre–B-ALL (Figure 4). Remarkable was the finding that 3 of 12 children with T-ALL even demonstrated a slight AS mRNA down-regulation, which would, in vitro, even point to sensitivity for L-Asp. Hyperdiploidy and the TEL/AML1 fusion are related to favorable outcome in childhood ALL,26–29 and are both in vitro–sensitive to L-Asp.18,30,31 In a previous study in TEL/AML1–positive ALL, Stams et al6 showed that TEL/AML1–positive children expressed 5-fold more AS mRNA compared with TEL/AML1–negative patients and healthy controls. In the present study, TEL/AML1 and hyperdiploid cases do not show an impaired in vivo up-regulation of AS that might have explained their high sensitivity to L-Asp. Taken together, both studies suggest that sensitivity to L-Asp as found in TEL/AML1–positive and hyperdiploid cells is not linked to decreased AS mRNA expression.

AS mRNA up-regulation in ALL cells occurs very rapidly (< 24 hours) after cellular asparagine depletion following PEG-Asp administration. Amino acids are required for protein synthesis, but they also play a role in the control of gene expression.5,21 The promoter of AS contains a nutrient-sensing response unit (NSRU) that is responsible for the induction of AS gene transcription upon amino acid deprivation.32 Iiboshi et al showed that withdrawal of asparaginase and glutamine by L-Asp resulted in a rapid inactivation of p70 S6 kinase.33 P70 S6 kinase participates in the mammalian target of rapamycin (mTOR) protein synthesis by controlling translational initiation and elongation factors as well as protein kinases that affect ribosomal assembly. Recently, gene expression profiling revealed that L-Asp–resistant ALL cells overexpressed several ribosomal protein-encoding genes as well as initiation factors.34 Using gene expression profiling, Fine et al showed that L-Asp–resistant cell lines expressed more baseline AS mRNA than sensitive leukemic cell lines, whereas no such association was found for primary pediatric ALL samples.35 This study emphasizes the fact that leukemic cell lines and primary samples from leukemic patients are different from each other and cell line data cannot be extrapolated to a primary patient’s cells that easily. Exposure to L-Asp altered in a primary patient’s samples the expression of a number of genes related to protein synthesis (ie, tRNA synthetases and amino acid transporters). However, no genes discriminative for L-Asp resistance in patient samples were found. These data point to a consistent coordinated response to amino acid starvation, which occurs irrespective of the level of resistance to L-Asp in a patient’s cells. Therefore, AS up-regulation may be a consequence of amino acid deprivation by L-Asp but is not the limiting key factor explaining resistance to L-Asp in pediatric ALL.

We conclude that up-regulation of AS mRNA in childhood ALL cells occurs within 24 hours after in vivo exposure to PEG-Asp, but these up-regulated levels are not associated with an early (poor) response to PEG-Asp in this small group of children.

References

Up-regulation of asparagine synthetase expression is not linked to the clinical response l-asparaginase in pediatric acute lymphoblastic leukemia

Inge M. Appel, Monique L. den Boer, Jules P. P. Meijerink, Anjo J. P. Veerman, Nathalie C. M. Reniers and Rob Pieters