Expression levels of asparagine synthetase in blasts from children and adults with acute lymphoblastic leukaemia

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Running title: *AS* levels in leukaemia

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Summary

L-Asparaginase is active in the treatment of acute lymphoblastic leukaemia (ALL) through the depletion of serum asparagine. Here we report that median *AS* mRNA levels were higher in acute myeloid leukaemia (AML) than ALL blasts in both children and adults, with intermediate levels in normal peripheral blood mononuclear cells (NPBMC). These results reached significance (one way ANOVA, $P<0.0001$); NPBMC versus child ALL (Tukeys Multiple Comparison Test, $P<0.05$); child ALL versus child AML ($P<0.001$) and adult ALL versus adult AML ($P<0.01$) and support the hypothesis that selectivity to treatment with L-asparaginase is due, at least in part, to lower *AS* expression.

Keywords

Asparagine synthetase, acute leukaemia, L-asparaginase, RQ-RT-PCR

L-asparaginase (L-asp) is an important component in the treatment of childhood acute lymphoblastic leukaemia (ALL). Recently the assumption that leukaemic cells are sensitive to L-asp due to a relative lack of asparagine synthetase (AS) has been questioned (Krejci*, et al* 2004, Stams*, et al* 2003). Using an RQ RT-PCR assay with transcription binding protein IID/TATA (TBP) as the internal control we provide evidence to support the original supposition that lymphoblasts have significantly lower levels of expression of *AS* than normal peripheral blood mononuclear cells (NPBMC).

Materials and methods

Following ethical approval bone marrow samples at diagnosis were obtained from 59 children (median 5 years; range 0.6-15.5) and 23 adults (median 29 years; range 17.7-71) with ALL and 8 children (median 5.2 years; range 1-16) and 22 adults (median 57; range 17-84) with acute myeloid leukaemia (AML). The presence of more than 80% blasts was confirmed by morphology. NPBMC were obtained from 14 healthy volunteers. Mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (Nycomed Pharma, Oslo, Norway). K562, MOLT-4 and NALM-6 cell lines were grown under standard conditions for use as controls.

Total mRNA was extracted using the RNeasy mini kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. Reverse transcription of 200ng total RNA was performed using a Taqman Reverse Transcription kit (Applied Biosystems) with random hexomers. Primers and Taqman probe for *AS* RQ RT-PCR, were designed using Primer-Express (Applied Biosystems). Primer and probe sequences were as follows; forward –5'-TCAGCCCGCCACATCAC-3'– (in exon1), reverse –5'-CAATGAAGCTATAAGCTTTCTTCAAGTG-3' (spanning exon 2 and 3), probe, –5'-CTGACCTGCTTACGCCCAGATTTTCTTCAA-3' (spanning exon one and two). Primers and probe for *TBP* were purchased as a Taqman pre-developed control reagent.

Standards containing 75, 18.75, 4.69, 1.17, 0.29 and 0.07ng of K562 total RNA with reverse transcribed E.coli tRNA as a carrier were assayed for *AS*

and *TBP* in triplicate on 27 separate occasions. CT values (the fractional cycle number at which the fluorescence passes the fixed threshold) for each standard were obtained using an ABI Prism 5700 sequence detection system and the average CT for each point plotted against log standard RNA concentration.

Reaction mixtures (82.5µl) contained 41.25µl Taqman PCR universal master mix, 900nM *AS* forward primer, 900nM *AS* reverse primer, 225nM *AS* probe or 4.13µl of ready synthesised *TBP* primers and probe and either 7.3µl of sample cDNA, 22.5µl of standard cDNA or 3.2µl of calibrator cDNA. Aliquots (25 µl) were added to wells of a microplate in triplicate. The thermal cycling conditions were 95^{o}C for 10 minutes, 40 cycles at 95^{o}C for 15 seconds and 60^{o}C for 1 minute.

A calibrator sample was quantified for *AS* and *TBP* in each assay. *AS* gene expression relative to the *TBP* gene were calculated as follows:

$$rAS = \frac{\text{Quantity of } AS \text{ sample / Quantity of } AS \text{ calibrator}}{\text{Quantity of } TBP \text{ sample / Quantity of } TBP \text{ calibrator}}$$

Results and Discussion

The reliability of the assay was determined by quantifying *AS* mRNA levels in MOLT-4, NALM 6 and K562 cell lines. Mean r*AS* values were 0.04, 0.50 and

0.84 respectively (n=7) Intra-assay coefficients of variation (CV) were 16.5% 13.4% and 10.1% (n=3). Inter-assay CV values were 32.1%, 9.04% and 9.97% (n=7). For childhood ALL, *rAS* values ranged from 0.013 to 0.84 (65-fold, median=0.17, n=59) and adult ALL 0.07 to 1.55 (22-fold, median=0.19, n=23) (figure 1). For childhood AML, *rAS* values ranged from 0.19 to 9.4 (49-fold, median=0.47, n=8) and adult AML 0.19 to 2.30 (12-fold, median=0.50, n=22). Little variation was observed in NPBMC where values ranged 0.27 to 0.49 (2-fold, median=0.40, n=14).

There was a higher median r*AS* in the AML patient samples than the ALL patient samples tested for both children and adults. The median r*AS* for NPBMC was higher than that for children and adults with ALL but less than that for children and adults with AML. These results reached significance (one way ANOVA following $\log_{10}$ transformation, $P<0.0001$ and as shown in figure 1). No significant difference in median r*AS* was observed comparing adults and children with either ALL or AML ($p>0.05$ in both cases)

No significant difference in r*AS* was detected between gender, immunophenotype, age at presentation or presenting white cell count for either adults or children with ALL or AML. No relationship was observed between r*AS* and cytogenetics, including the presence of the t(12;21) translocation or percentage blasts in the bone marrow at day 8 of therapy for children with ALL (data not shown).

In 1972 Horowitz and Meister (Horowitz and Meister 1972) suggested that leukaemic cells lack AS activity compared with normal cells and therefore rely on circulatory asparagine for survival. L-asp depletes asparagine from blood and bone marrow, starving the leukaemic cells of asparagine, causing them to undergo cell cycle arrest at G1 and ultimately apoptosis (Ueno*, et al* 1997). *In vitro*, resistance to L-asp has been shown to be highly correlated with cellular AS activity, mRNA and protein content (Hutson*, et al* 1997) and sensitive cell lines made resistant by repeated sub-culturing in sub-lethal doses of L-asp display increased *AS* expression (Kiriyama*, et al* 1989).

Our results contradict those reported by both Stams (2003) and Krejci (2004) in that we found that *AS* mRNA levels in NPBMC were significantly higher than those in lymphoblasts, in line with the expectation from many *in vitro* studies. As previously discussed (Krejci*, et al* 2005a, Krejci*, et al* 2005b, Stams*, et al* 2005) this may be due to the choice of the endogenous control used to normalize *AS* expression. We selected *TBP* as a suitable control as the variation of *TBP* expression between samples of lymphoid origin has been shown to be lower than either *GAPDH* or *β2microglobulin* (Lossos*, et al* 2003) and in our RQ RT-PCR assay we found that the abundance of *TBP* was similar to that of *AS*.

In summary, our observations support the original suggestion that *AS* expression is lower in lymphoblasts than in NPBMC and that this may explain their increased sensitivity to L-asp therapy.

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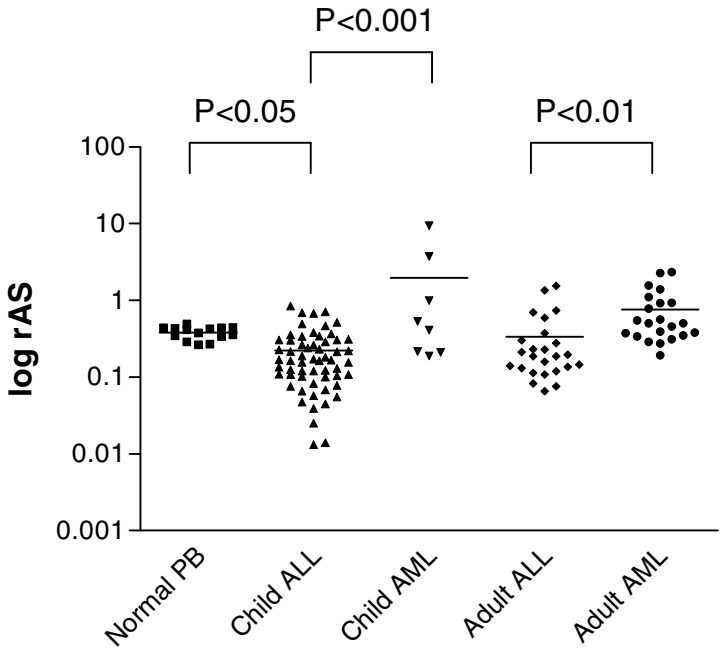
Title and legends to figures

Fig 1. Relative *AS* mRNA levels (rAS) of samples from children and adults with leukaemia and peripheral mononuclear cells from healthy individuals. Results are the average of three independent experiments with each sample quantified in triplicate. The significance of the comparisons indicated were calculated using Tukeys multiple comparison test.



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