Modulation of pyrimidine nucleotide synthesis in pediatric leukemia: the pivotal role of CTP synthetase

Verschuur, A.C.

Publication date
2000

Chapter 3

INCREASED ACTIVITY OF CYTIDINE TRIPHOSPHATE SYNTHETASE IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

A.C. Verschuur, A.H. van Gennip, E.J. Muller, P.A. Voûte
and A.B.P. van Kuilenburg

Laboratory of Genetic Metabolic Diseases and Department of Pediatric Oncology
Divisions of Pediatrics and Clinical Chemistry

Academic Medical Centre
University of Amsterdam
Emma Kinderziekenhuis AMC
P.O. Box 22700
1100 DE Amsterdam
THE NETHERLANDS

Advances in Experimental Medicine and Biology 1998; 373: 667-671

INTRODUCTION

Children suffering from acute lymphoblastic leukemia (ALL) possess an increased concentration of cytidine triphosphate (CTP) in their lymphoblasts compared to resting lymphocytes (1). This might be due to either an enhanced flux through the pyrimidine *de novo* and/or uridine salvage pathway or to an increased flux through the cytidine salvage pathway. By studying ribonucleotide fluxes in a MOLT-3 lymphoblastic cell line it has been shown that the increased CTP concentration is the result of an enhanced activity of CTP synthetase (CTPS) (2). We now analyzed the *in vitro* CTPS activity in lymphoblasts of children with ALL. If increased, CTPS might be inhibited by drugs like cyclopentenyl cytosine (CPEC).

PATIENTS AND METHODS

Cell isolation

The CTPS activity was measured in lymphoblasts of 16 pediatric patients with ALL at diagnosis and compared with proliferating and quiescent lymphocytes. Leukemic cells were obtained by bonemarrow aspiration of the posterior iliac crest or by venapuncture in case of a white blood cell count exceeding $100 * 10^6$ mononuclear cells/ml. The material was anticoagulated by EDTA. After collection, the cell suspension was diluted 1:1 with a "supplemented phosphate-buffered saline" (supplemented PBS) solution (9.2 mM Na_2HPO_4, 1.3 mM NaH_2PO_4, 140 mM NaCl, 5 mM glucose, 0.2% bovine serum albumin, 13 mM trisodiumcitrate and 5 mM EDTA, pH 7.4) and centrifuged (200 g, 10 min) after which the platelet-enriched plasma was discarded. The remaining cell pellet was again diluted 1:1 with supplemented PBS and was carefully layered on top of a 1.077 gr/mL Percoll solution with a 2:1 volume ratio. After centrifugation for 20 minutes at 800 g the interphase was collected and washed with supplemented PBS, after which the pellet was resuspended in 5 ml icecold erythrocyte-lysis buffer (8.29 gr/L NH_4Cl, 1.0 gr/L $KHCO_3$, 37.2 mg/L EDTA, pH 7.4); after 5 minutes of lysis 9 ml of supplemented PBS was added. The cells were washed twice and the final cell pellet was resuspended in a buffer containing PBS (9.2 mM Na_2HPO_4, 1.3 mM NaH_2PO_4, 140 mM NaCl, pH 7.4) and 5 mM glucose. Cells were counted with a Coulter Counter ZF cellcounter. Viability was assessed by the trypan blue exclusion test and cell purity was assessed morphologically by Jenner Giemsa staining and light microscopy.

Lymhocytes were isolated in the same way, but after collection of the mononuclear cells, lymphocytes were purified by magnetic cell sorting (Miltenyi, Germany), using CD11b microbeads or the pan-T isolation microbeads, and washed with PBS supplemented with 5 mM glucose. Proliferation of lymphocytes was achieved using round-bottom culture plates coated with anti-CD3 monoclonal antibody (CLB 16A9, 1:1000, 4 hr, 37°C), after which lymphocytes were cultured for 72 hours in RPMI 1640 containing 10% fetal bovine serum, 100 IU/ml penicilline, 100 µg/ml streptomycine, 200 µg/ml gentamycine, 0.125 µg/ml amphotericin B, 2 mM glutamine, 100 µM ß-mercaptoethanol and 50 U/ml Il-2.

CTP synthetase assay

The enzyme activity was measured by the method described by Van Kuilenburg (3) *et al.* Briefly, a cell homogenate was obtained by sonication, after which the homogenate was centrifuged and the supernatant was used for the assay. The protein content of the supernatant was determined with a modified Lowry method (4).

The assay-mixture contained 1 mM UTP, 4 mM ATP, 10 mM glutamine, 1 mM GTP, 20 mM MgCl2, 17 U/ml pyruvate kinase, 15 mM PEP, 10 mM dithiotreitol, 2.5 mM phenylmethylsulphonyl fluoride, 1 mM EGTA. After a 10 minute preincubation, the assay-mixture and cell homogenate were coïncubated for 2 hours, after which the nucleoside triphosphates were extracted by 0.55 N perchloric acid and neutralized with 0.35 M K_2CO_3. The nucleoside triphosphates were separated by anion-exchange high performance liquid chromatography (HPLC), under isocratic elution conditions using a 0.594 M NaH_2PO_4 buffer, pH 4.55. The retention times were 2.90, 4.15, 5.05 and 6.85 minutes for UTP, CTP, ATP and GTP respectively. Response factors were calculated using an external standard solution containing authentic nucleotides UTP, CTP, ATP and GTP. Absorption was measured at a wavelength of 280 nm.

The HPLC system consisted of a Gilson 231XL and 402 sampling device, a Perkin Elmer Binary LC 250 pump, a Whatman Partisphere SAX 4.6 x 125 mm column (5 µm particles) and Whatman 10 x 2.5 mm AX guard column, Waters UV detector, a Nelson 900 series Interface and Nelson PC Integrator Software version 5.1.5.

RESULTS

There were 11 cases of precursor B-lymphocytic leukemia (common ALL) and 5 cases

of T-lymphocytic leukemia. Ages ranged from 14 months to 15 years (mean 5.6 years). White blood cell counts ranged from 2.4 * 10^9/L to 610 * 10^9/L.
The purified leukemic cells of the patients showed a purity of at least 80%. The control lymphocytes were > 95% pure and > 90% viable.

The mean activity of CTP synthetase proved to be significantly higher in lymphoblasts compared to quiescent lymphocytes (6.8 versus 1.8 nmol CTP/mg protein/hr, p=0.002) (fig.1). The activity in lymphoblasts seemed slightly higher compared to proliferating lymphocytes although not yet significant (6.8 versus 5.0 nmol CTP/mg protein/hr, p=0.17) (fig.1).

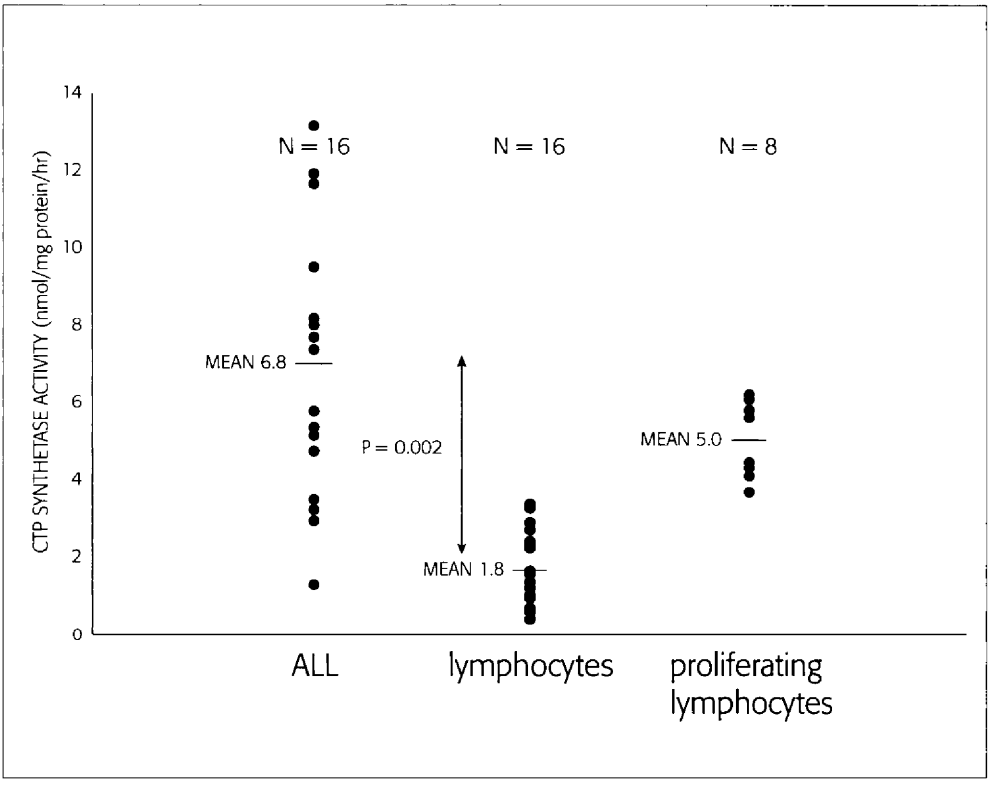

Figure 1: The specific CTPS activity is shown for ALL lymphoblasts (column 1), quiescent lymphocytes (column 2) and proliferating lymphocytes (column 3). The difference in CTPS activity between column 1 and 2 is significant (p = 0.002).

No correlation could be observed between the activity of CTP synthetase and the white blood cell count, nor with the percentage of lymphoblasts of the peripheral blood. A comparable mean enzyme activity was observed in T-ALL and B-ALL.

Preliminary results of incubation experiments with lymphoblasts of a patient with CPEC (kindly provided by the National Cancer Institute, Bethesda, Maryland) showed that CPEC is metabolized to it's active triphosphate form, which inhibited CTPS activity and led to a CTP depletion (fig.2).

At a CPEC concentration of 1.25 μM a CTP depletion was observed with an increased UTP concentration, reflecting inhibition of CTPS. At higher CPEC concentration (>5 μM) both CTP and UTP concentrations decreased, which could be attributed to competition of CPEC with uridine and cytidine at the uridine/cytidine kinase level, in addition to CTPS inhibition. Similar patterns were observed in two other patients.

CPEC incubation experiments with proliferating T-lymphocytes also showed a profound CTP depletion, which was paralelled by a decreased proliferation rate. Therefore, CTP synthetase plays an important role not only in malignant lymphoblasts but probably also in proliferating T-lymphocytes.

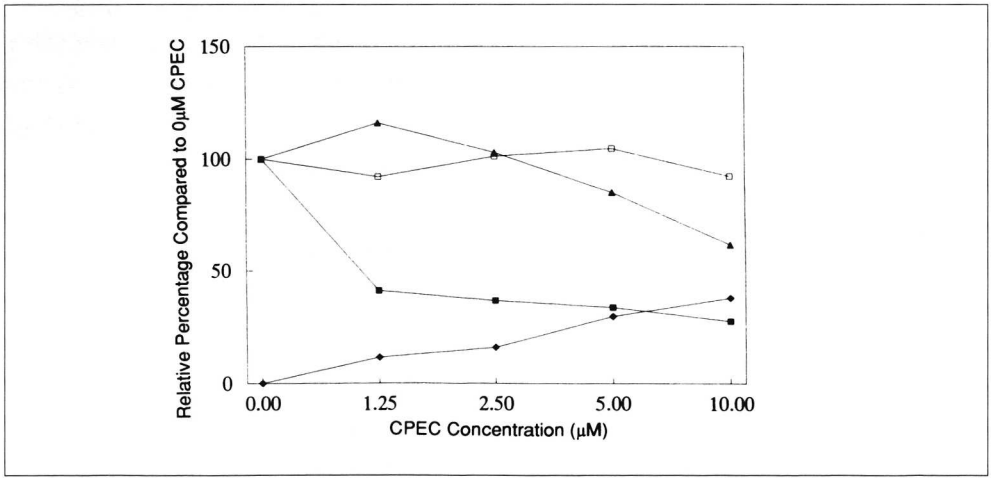

Figure 2: Incubation experiments of ALL lymphoblasts using increasing CPEC concentrations showing the effects on intracellular CTP (black squares), UTP (triangles), CPEC-TP (diamonds), and ATP (open squares) concentrations, depicted as the relative percentages of ribonucleotide concentrations compared to the control sample without CPEC.

DISCUSSION

A high CTP synthetase activity in lymphoblasts of pediatric patients is in line with studies of other types of malignancies (hepatoma, renal cell carcinoma, colon carcinoma,

lymphoma) (5-7). Adults suffering from ALL also showed an increased enzyme activity (7). However, the mean activity of CTPS observed in lymphoblasts of children with ALL was two-fold higher compared to that observed in lymphoblasts of adults with ALL (7). This phenomenon might be explained by the profound effect of protein concentration on the specific activity of CTPS (4).

Proliferating lymphocytes also showed a high CTPS activity, which might reflect the higher needs for nucleotides as their metabolism is accelerated to facilitate cell proliferation. Surprisingly, no correlation between the enzyme activity and the white blood cell count was observed in pediatric patients with ALL. This could suggest that the high enzyme activity is independent of the leukemic cells' proliferation rate, but it should be noted that lymphoblasts do not proliferate anymore in vitro.

CTPS inhibition was accomplished in our experiments since CTP depletion occurred in our patients' lymphoblasts treated with CPEC, concurrent with an increased UTP concentration. CPEC also seemed to cause CTP depletion in proliferating T-lymphocytes, suggestive of CTPS inhibition. However, a direct effect of CPEC on cytidine salvage in proliferating lymphocytes can not be excluded since the majority of CTP seems to be produced by salvage of cytidine in proliferating T-lymphocytes (2), and CPEC might compete with cytidine as a substrate for uridine/cytidine kinase.

CPEC is not only a potential cytotoxic drug, but is also capable of enhancing the cytotoxic effect of conventional drugs like arabinofuranosyl cytosine (AraC)(7), which is currently being used for the treatment of pediatric ALL. AraC must be phosphorylated to AraCTP in order to become cytotoxic, and a high dCTP concentration, that results from high CTP levels, will inhibit the first phosphorylation step of AraC (catalyzed by deoxycytidine kinase). Furthermore dCTP and AraCTP compete with each other in their affinity for DNA polymerase. Lowering the CTP concentrations by inhibition of CTPS, might therefore lead to lower dCTP levels and enhance the cytotoxic effect of AraC.

CONCLUSION

Our results provide the first evidence of an increased CTPS activity in pediatric ALL. Therefore, inhibiting CTPS by a drug like CPEC might be promising, and may cause a direct cytotoxic effect on leukemic cells, as well as a modulating effect on the cytotoxicity of AraC.

ACKNOWLEDGEMENT

The authors wish to express their gratitude for the generous financial support provided by the Dutch Cancer Society and the Stichting Kindergeneeskundig Kankeronderzoek.

REFERENCES

1 **De Korte D, Haverkort WA, Roos D, Behrendt H, Van Gennip AH.** Imbalance in the ribonucleotide pools of lymphoid cells from acute lymphoblastic leukemia patients. Leukemia Res 1986; 10: 389-396.

2 **Van den Berg AA, Van Lenthe H, Busch S, De Korte D, Roos D, Van Kuilenburg ABP, Van Gennip AH.** Evidence for transformation-related increase in CTP synthetase activity in situ in human lymphoblastic leukemia. Eur J Biochemistry 1993; 216: 161-167.

3 **Van Kuilenburg ABP, Elzinga L, Verschuur AC, Van den Berg AA, Slingerland RJ, Van Gennip AH.** Determination of CTP synthetase activity in crude cell homogenates by a fast and sensitive non-radiochemical assay using anion-exchange HPLC. J Chromatogr B 1997; 639: 287-295.

4 **Peterson GL.** A simplification of the protein assay method of Lowry et al which is more generally applicable. Anal Biochem 1977; 83: 346-356.

5 **Williams JC, Kizaki H, Weber G.** Increased CTP synthetase activity in cancer cells. Nature 1978; 271: 71-72.

6 **Kizaki H, Williams JC, Morris HP, Weber G.** Increased Cytidine 5'- triphosphate synthetase activity in rat and human tumors. Cancer Res 1980; 40: 3921-3927.

7 **Ellims PH, Gan TE, Medley G.** Cytidine triphosphate synthetase activity in lymphoproliferative disorders. Cancer Res 1983; 43: 1432-1435.

8 **Lillemark JO, Plunkett W.** Regulation of 1-ß-D- Arabinofuranosylcytosine 5-triphosphate accumulation in human leukemia cells by deoxycytidine 5-triphosphate. Cancer Res 1986; 46: 1079-1083.