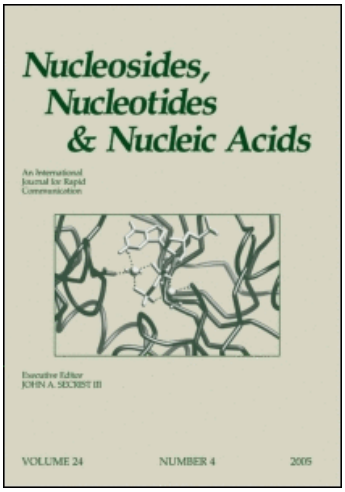

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[a] Department of Pediatric Hematology/Oncology, VU University Medical Center, Amsterdam, The Netherlands [b] Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands [c] Institute of Molecular Biology and Biotechnology, Herakleion, Crete, Greece [d] Dutch Childhood Oncology Group (DCOG), The Hague, The Netherlands [e] MRC Childhood Leukaemia Working Party, Glasglow, UK [f] AML-BFM Study Group, Münster, Germany

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Immunocytochemical Detection of Deoxycytidine Kinase in Pediatric Malignancies in Relation to In Vitro Cytarabine Sensitivity

I. Hubeek,[1,*] G. J. Peters,[2] A. J. F. Broekhuizen,[1] I. Talianidis,[3] A. Y. N. Schouten van Meeteren,[1] E. R. van Wering,[4] B. Gibson,[5] U. Creutzig,[6] and G. J. L. Kaspers[1]

[1]Department of Pediatric Hematology/Oncology and
[2]Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands
[3]Institute of Molecular Biology and Biotechnology, Crete, Greece
[4]Dutch Childhood Oncology Group (DCOG), The Hague, The Netherlands
[5]MRC Childhood Leukaemia Working Party, Glasgow, UK
[6]AML-BFM Study Group, Münster, Germany

ABSTRACT

Deoxycytidine kinase (dCK) is essential for the phosphorylation of cytarabine (ara-C), a deoxycytidine analog active against acute leukemias. Resistance to ara-C has been linked to dCK deficiency. In this study we determined the expression of the dCK protein in pediatric malignancies, using immunocytochemistry and related the expression levels to in vitro ara-C sensitivity (measured with the MTT-assay). dCK expression was high in the AML and retinoblastoma samples, in the ALL samples dCK expression ranged from low to very high. The brain tumor samples expressed low levels of dCK. AML was significantly more sensitive in vitro to ara-C compared to ALL ($p = 0.03$). Retinoblastoma and brain tumor cells were extremely resistant in

*Correspondence: I. Hubeek, Department of Pediatric Hematology/Oncology, VU University Medical Center, Amsterdam, The Netherlands; E-mail: isabelle.hubeek@vumc.nl.

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vitro, we were unable to detect more than 50% ara-C induced cell kill in the majority of samples. Samples were combined in groups according to dCK expression. Samples with low dCK expression were significantly more resistant to ara-C compared to samples with high dCK expression. In conclusion, dCK expression varies between individual samples and between different types of malignancies and may play a role in resistance to ara-C in particular tumor types.

Key Words: Deoxycytidine kinase; Immunocytochemistry; Pediatric malignancies.

INTRODUCTION

The pyrimidine nucleoside analog 1-β-D-arabinofuranosylcytosine (ara-C, cytosine arabinoside) is the most effective drug for the treatment of acute myeloid leukemia (AML).[1] It is also active in other hematological malignancies, including acute lymphoblastic leukemia (ALL).[2] The effectiveness of ara-C depends on the formation of its active metabolite, the triphosphate ara-CTP, by deoxycytidine kinase (dCK), and successive phosphorylations by dCMP kinase and dCDP kinase, respectively.[3] dCK deficiency has been linked to resistance to ara-C.[4] In addition, dCK also plays a crucial role in the activation of other clinically important nucleoside analogs like cladribine and gemcitabine.[1] In vitro ara-C sensitivity has been described previously for childhood acute leukemias, retinoblastoma and brain tumors. Whereas leukemia samples were more sensitive to ara-C, retinoblastoma and brain tumor samples were extremely resistant in vitro.[5–7] This difference in ara-C sensitivity might be due to differences in the expression of dCK in these malignancies. In this study we have evaluated the expression of the dCK protein using a novel immunocytochemistry method in AML, ALL, retinoblastoma and brain tumors and related differences in dCK expression levels to the in vitro sensitivity to ara-C.

MATERIALS AND METHODS

Patient Samples

At initial diagnosis, bone marrow or peripheral blood samples from children with AML (n = 50) or ALL (n = 49) were collected, with informed consent for additional research studies. Samples were provided by several groups: 1) DCOG, The Hague, The Netherlands; 2) MRC Working Group, UK and 3) The AML-BFM Study Group, Münster, Germany. Central review of diagnosis, clinical data, and FAB classification were performed by reference laboratories of these groups. Mononuclear cells were isolated by density gradient centrifugation (Ficoll Paque, density 1.077 g/ml; Pharmacia, Sweden) and re-suspended in culture medium. The percentage of leukemic cells was determined morphologically (May-Grünwald-Giemsa staining (MGG); Merck, Darmstadt, Germany). In case of a low blast percentage (<80%) enrichment procedures were performed as described previously.[8] Primary tumor samples (8 retinoblastoma and 11 brain tumors, of which 7 primitive neuro-ectodermal tumors and 4 ependymoma) were collected fresh and under sterile conditions as described previously.[6,7]

Cytospins were prepared by centrifugation of 50 µl of cell suspension (0.5×10^6 cells/ml PBS containing 5% human serum albumin) at 50 g for 7 minutes. Cytospins were air-dried on silica gel for at least 48 hours and subsequently stored at −20°C.

Immunocytochemistry

Cryopreserved cytospins were thawed at room temperature for 30 minutes. Cells were fixed (10% formaldehyde/20 minutes) and endogenous myeloperoxidase was inactivated (PBS/1%H_2O_2 for 10 minutes). Slides were washed with PBS and blocked with 10% normal swine serum (Sigma, St Louis, USA) for 30 minutes. The slides were incubated with rabbit-anti human dCK antibody diluted 1:200 in 1% bovine serum albumin (BSA)/10% pooled human serum (PHS)/PBS for 2 hours. After PBS wash steps, the secondary antibody, biotinylated swine anti-rabbit (1:300 diluted in PBS// 1%BSA/2%PHS); DAKO, Glostrup, Denmark), was applied for 30 minutes. Slides were rinsed with PBS and incubated with horseradish peroxidase-conjugated streptavidin for 45 minutes (1:100 diluted in PBS/1%BSA; DAKO, Glostrup, Denmark). Peroxidase activity was determined using 1 mM 3,3′diaminobenzidine (DAB, Sigma, St. Louis, USA), 0.05 M imidazole (Merck, Darmstadt, Germany) and 0.036% H_2O_2 in 0.05 M Tris Buffer (pH 7.4) for 10 minutes. Finally, cells were counterstained using Mayer's Haematoxylin Solution (Merck, Darmstadt, Germany) and embedded in malinol. As a positive control the dCK positive cell line CCRF-CEM was used in each experiment. Negative controls were performed for each sample by omitting the primary antibody. The staining was examined using a light microscope by two independent observers (IH and AJFB). dCK protein expression was determined by semi-quantitative scoring of the intensity of the staining (negative = 0, low = 1+, intermediate = 2+, high = 3+, and very high = 4+).

MTT Assay

In vitro drug resistance was determined using the 4 day total cell kill MTT assay, as described previously.[8] Briefly, isolated tumor cells were incubated with six concentrations of ara-C (0.04–41.1 µM for the leukemic samples and 0.03–1032.0 µM for the retinoblastoma and brain tumor samples (Cytosar®; Pharmacia & Upjohn, Woerden, The Netherlands) for 4 days in 5% CO_2 humidified air at 37°C. On day 4 10 µl of 3-(4,5-dimethylthiazol-2,5-diphenyl) tetrazolium bromide (MTT; Sigma, St Louis MO, USA) was added to each well, shaken and incubated for 6 hours. Only living cells are able to reduce MTT to purple coloured formazan. Formazan crystals formed were dissolved with acidified isopropanol. The optical density (OD) was measured in a ELISA EL-312 microtiter spectrophotometer (Biotek instruments, USA) at wave lengths of 562 and 720 nm. The OD correlates linearly with the amount of viable cells.[9] The LC50 value, the concentration of drug lethal to 50% of the tumor cells, was calculated from the dose-response curve.

Statistics

Differences in the distribution of variables between groups were analyzed by the Chi-square test and the Mann-Whitney U (MWU) test. Analyses were two-tailed and

Table 1. Expression of the dCK protein [number of patients (%)] and in vitro sensitivity to ara-C in different pediatric malignancies, determined by immunocytochemistry.

	dCK immunocytochemistry						
	n	0	1+	2+	3+	4+[a]	p-value[b]
AML	50			18 (36.0)	26 (52.0)	6 (12.0)	
ALL	49		10 (20.4)	11 (22.4)	18 (36.7)	10 (20.4)	0.003*
Retinoblastoma	8		1 (12.5)	2 (25.0)	4 (50.0)	1 (12.5)	0.09
Brain tumors	11	1 (9.1)	3 (27.3)	7 (63.6)			<0.0001*

[a]0 = negative; 1+ = low; 2+ = intermediate; 3+ = high and 4+ = very high.
[b]p-value in comparison to AML; determined with the Chi-square.
*Indicates significant p-value.

differences were considered statistically significant when $p \leq 0.05$ (SPSS 9.0, Inc., Chicago, Il).

RESULTS

We observed clear differences in dCK expression between individual patients, as well as between different tumor types. The results are summarized in Table 1. dCK expression was highest in the AML and retinoblastoma samples, the majority of these samples expressed high levels of dCK, 52 and 50%, respectively. The ALL and brain tumor samples expressed lower levels of the dCK protein in comparison to AML blasts ($p = 0.003$ and $p < 0.0001$, respectively), and one brain tumor sample was completely negative. AML blasts were most sensitive in vitro to ara-C, significantly more sensitive than ALL blasts ($p = 0.03$), retinoblastoma samples ($n = 8$) and brain tumor samples ($n = 11$). In 6/8 retinoblastoma samples and 7/11 brain tumor samples we were unable to detect an LC50 value with the drug concentrations tested, less than 50% of cells were killed at the highest concentration of ara-C (Table 2). All samples were combined in groups according to the level of dCK expression and analysed whether the level of

Table 2. In vitro sensitivity to ara-C (μM), determined with the MTT assay in pediatric malignancies.

	n	Median LC50 value ara-C (25th–75th percentile; μM)	p-value[a]
AML	50	1.79 (0.80–2.47)	–
ALL	49	2.98 (0.97–5.71)	0.03*
Retinoblastoma	8	>1032	<0.0001*
Brain tumors	11	>1032	<0.0001*

[a]p-value in comparison to AML; determined with the MWU test.
*Indicates significant p-value.

dCK expression was related to in vitro ara-C sensitivity. The group with a low dCK expression was significantly more resistant to ara-C in comparison with the group with a high dCK expression ($p = 0.03$, Fig. 1).

DISCUSSION

In a group of 118 pediatric tumor samples (AML, ALL, retinoblastoma and brain tumors) dCK protein expression was related to in vitro sensitivity to ara-C. Cells that expressed low levels of dCK were significantly more resistant to ara-C in vitro compared to cells with a high expression level of dCK. dCK expression in AML blasts ranged from intermediate to very high, with the majority of samples expressing high levels of dCK (52%). Moreover, we did not observe any AML sample with a low expression of the dCK protein. AML blasts were most sensitive to ara-C in vitro in comparison to the other tumor types. We did not observe a relation between dCK expression and ara-C sensitivity in AML (data not shown). This might indicate that in AML blasts dCK expression is sufficiently high and other cellular mechanisms contribute to resistance ara-C. ALL blasts expressed significantly lower levels of the dCK protein in comparison to AML and were 1.7-fold more resistant to ara-C in vitro. Brain tumor samples expressed very low levels of dCK, and were extremely resistant to ara-C in comparison to both AML and ALL. In ALL and brain tumor cells dCK protein levels might play a role in ara-C sensitivity. Retinoblastoma cells expressed high levels of dCK, comparable to AML blasts, but were extremely resistant in vitro. dCK expression can therefore not explain resistance to ara-C in retinoblastoma. However, only a relatively low number of patients could be evaluated. One of the major limitations of this study is that we were unable to measure dCK activity, due to lack of sufficient sample of each patient. However, dCK protein levels have previously been shown to be related to dCK activity.[10] In conclusion, dCK expression varies between

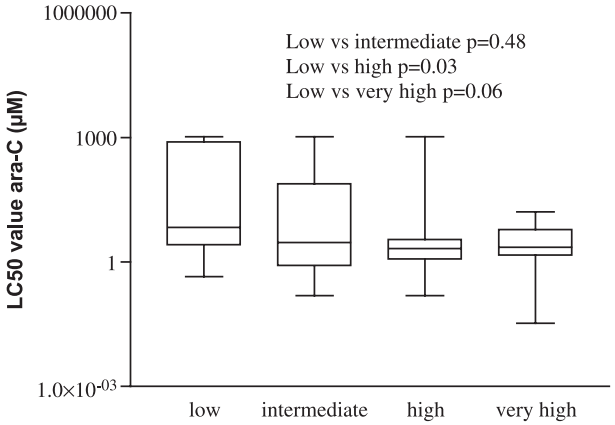

Figure 1. Expression of dCK and in vitro ara-C sensitivity. AML, ALL, retinoblastoma and brain tumor samples were pooled and divided according to the level of dCK expression. P-values determined with MWU test.

individual samples and between different types of malignancies and may play a role in resistance to ara-C in particular tumor types, e.g. ALL and brain tumors. In AML however dCK expression was high and other factors appear to contribute to in vitro sensitivity to ara-C.

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