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The pattern of deoxycytidine- and deoxyguanosine kinase activity in relation to messenger RNA expression in blood cells from untreated patients with B-cell chronic lymphocytic leukemia

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ABSTRACT

Deoxycytidine kinase (dCK) and deoxyguanosine kinase (dGK) catalyze the first step in the intracellular cascade of fludarabine (2-fluoroadenine-β-D-arabinofuranoside) and cladribine (2-chlorodeoxyadenosine) phosphorylation, which leads to activation of these prodrugs, commonly used for treatment of chronic lymphocytic leukemia (CLL). Thus, resistance to nucleoside analogues may primarily be due to low levels of deoxynucleoside kinase activity.

The purpose of this study was to investigate the activity profiles of dCK and dGK and characterize the possible relationship between the levels of dCK enzymatic activities and mRNA levels in B-CLL cells from untreated patient samples in an attempt to determine the best approach for predicting sensitivity to nucleoside analogues and thereby optimizing treatment of CLL. For this purpose, dCK and dGK analyses were done in blood cells from 59 untreated symptomatic patients with CLL. The dGK activity towards 2-chlorodeoxyadenosine was significantly lower than of dCK (median 73 pmol/mg protein/min (85–121, 95% CI) versus 353 pmol/mg protein/min (331–421)). The median dCK mRNA level was 0.107 (0.096–0.120, 95% CI). There was a lack of correlation between the activities of dCK and dGK, which indicates that these proteins are regulated independently. We also found that the dCK and dGK activity measurement towards their endogenous substrates were comparable to the nucleoside analogues tested. Such variations in enzyme activities and mRNA levels may well explain differences in clinical responses to treatment.

There was no correlation between the levels of dCK mRNAs and enzymatic activities using a quantitative real-time PCR procedure. Sequencing of dCK mRNA did not reveal alternate splicing or mutations in the coding region. The relation between activity and mRNA levels was studied by short interfering RNA (siRNA) method, which showed that in

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the siRNA treated cells the down-regulation of dCK expression, and activity followed each other. However, in control cells the mRNA levels remained stable but the protein activity markedly decreased. These data demonstrate that the dCK activity is not reflected by dCK mRNA expression that indicates a post-translational mechanism(s).

1. Introduction

Heterogeneity in the clinical behavior of chronic lymphocytic leukemia (CLL), the most common type of leukemia in Europe and North America, makes it difficult to accurately identify patients who may benefit from first-line treatment with chlorambucil or from a more modern treatment strategy involving the purine analogues fludarabine (2-fluoroadenine-β-D-arabinofuranoside, Fara-A) or cladribine (2-chlorodeoxyadenosine, CdA). Unfortunately, the mechanisms underlying resistance to these drugs are largely unknown in most cases. 2-fluoroadenine-β-D-arabinofuranoside and 2-chlorodeoxyadenosine which have been proven to be valuable in treating CLL [1–4], are prodrugs and converted to their active forms intracellularly through the action of the nuclear/cytosolic enzyme deoxycytidine kinase (dCK) [5] and, possibly, of the mitochondrial enzyme deoxyguanosine kinase (dGK) as well [6]. The cytotoxicity of these drugs is exerted by their 5′-triphosphate derivatives. dCK exhibits a broad substrate specificity phosphorylating many endogenous substrates including deoxycytidine (dCyt), deoxyadenosine, deoxyguanosine (dGvo) and nucleoside analogues used as important clinical drugs such as cladribine, fludarabine, cytarabine (Ara-C), gemcitabine, nelarabine (AraG) and clofarabine. Pre-treatment of both normal as well as leukemic lymphocytes with a variety of compounds has been reported to increase their dCK activity probably through post-translational modifications [7–10]. dGK phosphorylates the natural substrate deoxyguanosine as well as important nucleoside analogues [6,11].

Although the cause of resistance to nucleoside analogues in CLL has not yet been elucidated, considerable evidence from in vitro studies suggests that the levels of these phosphorylating enzymes play an important role in this context. Accordingly, the purpose of the present investigation was to analyze the enzymes involved in the metabolism of purine nucleoside analogues in blood cells from patients with chemo-naïve symptomatic B-CLL. To this end, we employed a radiochemical-based procedure for determination of enzyme activity and a real-time quantitative polymerase chain reaction for quantitating the corresponding level of mRNA.

2. Materials and methods

2.1. Drugs, chemicals and other reagents

2-chlorodeoxyadenosine was synthesized by Dr. Zygmunt Kazimierczuk at the Foundation for the Development of Diagnostics and Therapy (Warsaw, Poland) and 2-fluoroadenine-β-D-arabinofuranoside was a kind gift from Dr. Zeev Shaked (Berlex, Alameda, CA). 9-β-D-arabinofuranosylguanine (R.I. Chemical, Orange, CA), [5-^{3}H(N)]-deoxycytidine, 16.7 Ci (618 GBq)/mmol, [8-^{3}H]-2-chlorodeoxyadenosine, 4 Ci (148 GBq)/mmol, [8-^{3}H]-9-β-D-arabinofuranosylguanine, 2 Ci (74 GBq)/mmol and [8-^{3}H]-2-fluoroadenine-β-D-arabinofuranoside, 15 Ci (555 GBq)/mmol (Moravek Biochemicals, Brea, CA), [5-^{3}H]-cytarabine 28 Ci (1040 GBq)/mmol (Amersham Little Chalfont, England) and RPMI 1640 medium, heat-inactivated fetal calf serum, L-glutamine, and penicillin–streptomycin (Gibco, Life Technologies, Paisley, UK) were obtained from sources indicated. All reagents for PCR reactions were purchased from Perkin-Elmer (Foster City, CA) and all other reagents were from Sigma Chemical Co. (St. Louis, MO).

2.2. Isolation of peripheral leukocytes from patients with B-CLL

Blood samples were obtained from patients participating in a Phase III International Study randomizing between chlorambucil, fludarabine and 2-chlorodeoxyadenosine as the primary treatment for B-CLL with including centers in Scandinavia and Australia. Prior to any drug treatment, peripheral blood mononuclear cells were isolated from 59 patients employing density gradient centrifugation on Lymphoprep® (Nycomed, Oslo, Norway) and thereafter vitally frozen in liquid nitrogen for later analysis. The study was approved by the regional ethics committee for human research at Linköping University.

2.3. Assay of enzymes involved in the metabolism of purine analogues

The kinases dCK and dGK were assayed in crude extracts employing deoxycytidine (10 μM), deoxyguanosine (50 μM), cytarabine (80 μM), 2-chlorodeoxyadenosine (50 μM), 2-fluoroadenine-β-D-arabinofuranoside (500 μM) and 9-β-D-arabinofuranosylguanine (80 μM) as substrates all at specific radioactivities of 500–1000 cpm/pmol, essentially as described previously [12]. Briefly, the frozen cells were thawed and then washed with PBS. The pellet was extracted in a buffer containing 50 mM Tris–HCl, pH 7.6, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol and 0.5% Nonidet P40. Thereafter the resuspended cells were frozen and thawed three times and centrifuged at 11,800 × g for 5 min at 4 °C in order to remove cell debris. The protein in the supernatant thus obtained was used in the kinase assays, and the concentration was determined by the DC protein assay (Bio-Rad Laboratories, CA), according to the manufacture's instructions.

Subsequently, the activity of 2–3 μg of protein was assayed in a reaction mix containing 50 mM Tris–HCl (pH 7.6), 5 mM magnesium chloride, 5 mM adenosine triphosphate, 4 mM dithiothreitol, 10 mM sodium fluoride and substrate in a total volume of 25 μl.

The concentration for each substrate employed (specified above) was approximately 10-fold higher than the K_m value for the principle kinase phosphorylating respective substrate. Following the assay, 10 μl of the reaction mix was spotted onto Whatman DE81 filters and radioactivity determined employing 3 ml Ecoscint scintillation fluid in a liquid scintillation counter (RackBeta; LKB Wallac, Turku, Finland). The radiolabeled substrates were dissolved in 50% methanol, dried under nitrogen and redissolved in PBS prior to use. Determination of dGK activity towards 9-β-D-arabinofuranosylguanine and 2-chlorodeoxyadenosine was carried out in the presence of an excess deoxycytidine (1 mM) to inhibit dCK.

2.4. RNA extraction and cDNA synthesis

The cells were first thawed and washed in PBS after which total RNA was extracted utilizing the RNA Blood Mini Kit (Qiagen, VWR International AB, Stockholm, Sweden). The concentration and the quality of the total RNA thus obtained were determined by chip-based electrophoresis using an Agilent 2100 bioanalyzer, with samples for which the ratio of 28S to 18S rRNA exceeded 1.7 being considered of high quality. Double-stranded complementary DNA (cDNA) synthesis was performed with 1 μg of total RNA in a 100 μl reaction mixture with the TaqMan® Reverse Transcription Kit (Applied Biosystems, Stockholm, Sweden), in a Mastercycler Personal (Eppendorf, Hamburg, Germany) using Random Hexamers as primers.

2.5. Real-time quantitative PCR (RQ-PCR)

The primers and probes for dCK and β_2-microglobulin, used as an internal control were designed as documented in Table 1 and synthesized by Invitrogen AB (Lidingö, Sweden), Applied Biosystems (Stockholm, Sweden) or Scandinavian Gene Synthesis AB (Köping, Sweden). The 5′-ends of the probes were labeled with the reporter dyes FAM in the case of dCK and VIC for β_2-microglobulin. TAMRA was incorporated as a quencher at the 3′-end of the probes for β_2-microglobulin while a dark quencher was inserted into the dCK probe. All reactions were performed in duplicate run on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The 25 μl amplification mixture contained 2× TaqMan® Universal PCR Master Mix (1×), forward and reverse primers (0.8 μM), probes (0.2 μM) and cDNA (10 ng/reaction). The reaction conditions were as follow: 2 min at 50 °C; 10 min at 95 °C; 40 cycles with 15 s at 95 °C and 1 min at 60 °C.

2.6. Reverse transcription-PCR (RT-PCR)

Separation and visualization of the PCR products obtained from dCK mRNA were accomplished with the Agilent 2100 Bioanalyzer in conjunction with the DNA 1000 LabChip® kit for the detection of DNA fragments with a maximum size of 1000 bp [13] according to the manufacture's instructions. Twelve samples on each chip were analyzed in an automated run lasting approximately 30 min, resulting in the sizes and relative concentrations of the different PCR products obtained from dCK mRNA. Using two specific primers for intact dCK mRNA, A7 and B5 [14,15], cDNA was then synthesized from this mRNA by reverse transcription.

2.7. Silencing of dCK with short interfering RNA (siRNA)

CEM cells were used for silencing of mRNA and subsequently the dCK activity. Cells were washed in RPMI-1640 media, resuspended in RPMI, to a concentration of 4×10^6 cells/ml and 1.25% DMSO was added to enhance cell survival and transfection efficiency. Cells were electroporated using a Gene Pulser Xcell Electroporation System (Bio-Rad Laboratories, CA). Parameter settings were square waves, 340 V, two pulses of 10 ms with 10 s between in a 4 mm cuvette. Small interfering RNA used was the *Silencer*™ Validated siRNA#69 for human deoxycytidine kinase (Ambion, Inc., Huntington, Cambridge, UK). Deoxycytidine kinase siRNA and GADPH siRNA used to control for any toxic effects of the siRNA were used in a concentration of 250 nM. Mock transfected cells

Table 1 – Primers and probes used in the PCR reactions

	Sequence	bp
Real-time PCR		
dCK		
Forward primer	5′-CCACCCCGCCCAAGA-3′	15
Reverse primer	5′-CTTCCCTGCAGCGATGTTC-3′	19
Probe	5′-TCTTTC**X**CAGCCAGCTCTGAGGGGACCT-3′	27
β_2-Microglobulin		
Forward primer	5′-AGGACTGGTCTTTCTATCTCTTGTACTACAC-3′	31
Reverse primer	5′-AAGTCACATGGTTCACACGGC-3′	21
Probe	5′-TCACCCCCAC**X**GAAAAAG ATGAGTATGCCT-3′	29
PCR		
Long dCK		
Forward primer	5′-ACACCATGGCCACCCCGCCCAAGAGAAGCT-3′	30
Reverse primer	5′-CACGGATCCTCACAAAGTACTCAAAAACTCTTT-3′	33
A7	5′-TCTTTGCCGGACGAGCTCTG-3′	20
B5	5′-TGGAACCATTTGGCTGCCTG-3′	20

'**X**' denotes the position of the internal dark quencher.

(e.g. electroporated without siRNA) were included to correct for any natural fluctuations over time of dCK mRNA or dCK activity. After electroporation cells were grown in RPMI-1640 media substituted with 20% fetal calf serum and 2 mmol/l L-glutamine and harvested every 24 h and assayed for dCK expression and activity.

2.8. Analysis of mutations by sequencing of dCK cDNA

A total of 15–30 ng of purified PCR product obtained from dCK mRNA was sequenced employing the ABI PRISM® 3700 DNA Analyzer with the BigDye® Terminator v3.1 Cycle Sequencing Kit according to recommendations of Applied Biosystems. Specific dye labeled nucleotides were used to terminate the primer extension step for each nucleotide.

2.9. Statistical analysis

Statistical analysis was performed by use of GraphPad Prism 4.0 software. The data were expressed as median. Non-parametric Spearman's rank correlation test was utilized to compare the relationships between dCK and dGK activity and mRNA levels. A p-value of ≤ 0.05 was considered to be statistically significant.

3. Results

3.1. Determination of enzymatic activity of dCK and dGK in leukocytes from patients with CLL

In order to determine the interindividual variability in the activities of the phosphorylating kinases, dCK and dGK, we assayed these enzymes using deoxycytidine and deoxyguanosine as substrates in leukocyte preparations from 53 patients (6 missing due to insufficient material). dGK was assayed in the presence of an excess of deoxycytidine and thymidine to inhibit dCK and the mitochondrial thymidine kinase. The activity of dCK towards deoxycytidine varied from undetectable to 695 pmol/mg protein/min (i.e. >200-fold), while dGK activity with deoxyguanosine ranged from 8 to 216 pmol/mg protein/min (27-fold) (Fig. 1). The median activity of dCK towards deoxycytidine was 145 pmol/mg protein/min, while dGK activity with deoxyguanosine was significantly lower 58 pmol/mg protein/min (Fig. 1; Table 2). The activities of dCK towards 2-chlorodeoxyadenosine, cytarabine, deoxycytidine and 2-fluoroadenine-β-D-arabinofuranoside and of dGK with 2-chlorodeoxyadenosine and 9-β-D-arabinofuranosylguanine (in the presence of excess deoxycytidine) exhibited good correlation with one another, as well as with the activities towards deoxycytidine and deoxyguanosine, respectively ($r^2 \geq 0.65$; Fig. 2A–D).

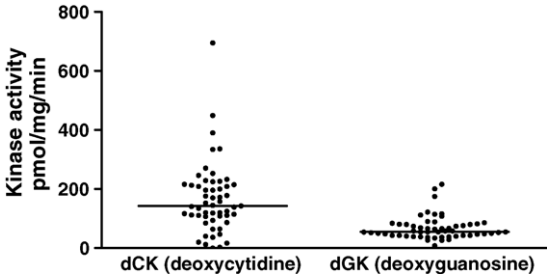

Fig. 1 – Large interindividual variations in the dCK and dGK activity of peripheral leukocytes from patients with CLL. Peripheral leukocytes were isolated from 53 B-CLL patients prior to initiation of chemotherapy and their dCK and dGK activities towards deoxycytidine (in the presence of excess thymidine) and deoxyguanosine (in the presence of excess deoxycytidine) as substrates, respectively, assayed. The median dGK activity (55 pmol/mg protein/min) was significantly lower than that of dCK (143 pmol/mg protein/min).

3.2. Analysis of the levels of dCK mRNA by RQ-PCR and correlation of these levels to enzyme activities

Of the methods for detection and quantification of mRNA presently available, real-time quantitative PCR is the most sensitive. In order to determine the relative levels of mRNA encoding dCK in our samples, we constructed specific primers and fluorogenic probes for these mRNA species, as well as for β_2-microglobulin mRNA [16] as an internal control (Table 1).

Table 2 – Quantitative mRNA and activity of dCK, dGK and thymidine kinase in peripheral blood cells from CLL patients

Substrate	No. of patients	Median	Lower 95% CI	Upper 95% CI
dCK, mRNA[a]	45	0.107	0.096	0.120
dCK activity[b]				
CdA	53	353	331	421
dCyt	53	145	139	204
Ara-C	53	193	187	259
Fara-A	53	152	143	176
dGK activity[b]				
CdA in excess of dCyt	53	73	85	123
dGuo in excess of dCyt	53	58	59	81
AraG in excess of dCyt	53	28	27	38

[a] Quantitative.
[b] pmol/mg protein/min.

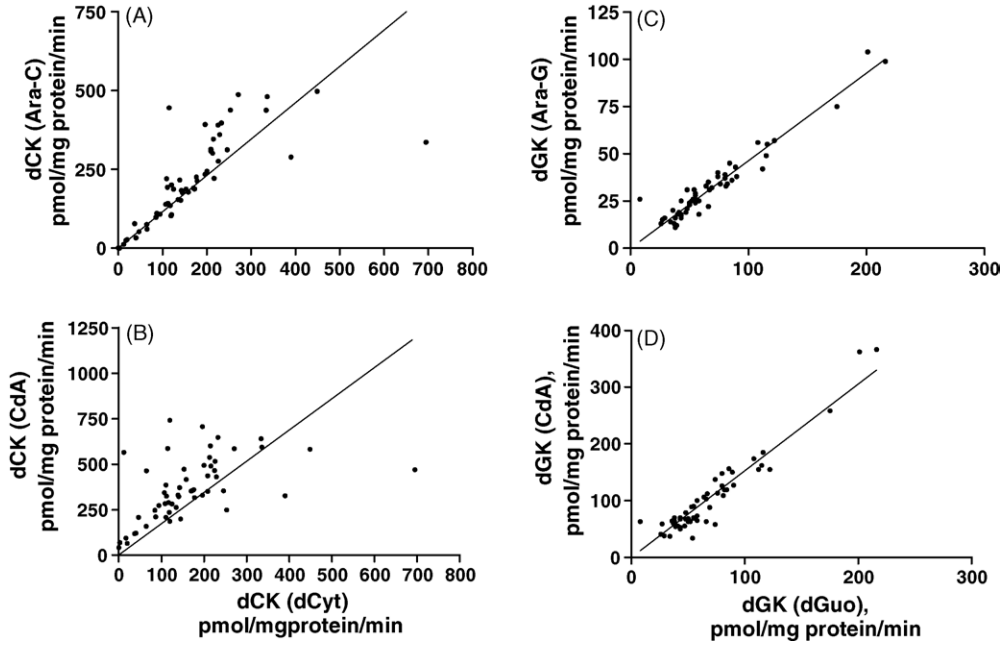

Fig. 2 – Correlation of the activities of dCK and dGK in peripheral leukocytes from CLL patients with different substrates. Peripheral leukocytes were isolated from B-CLL patients prior to initiation of drug treatment ($n = 55$). (A) Comparison of deoxycytidine (dCyt) and cytarabine (Ara-C) as substrates for dCK ($r = 0.90$, $p < 0.0001$). (B) Comparison of deoxycytidine (in presence of excess thymidine) and 2-chlorodeoxyadenosine (CdA) as substrate for dCK ($r = 0.64$, $p < 0.0001$). (C) Comparison of deoxyguanosine (dGuo) and 9-β-D-arabinofuranosylguanine (AraG) (in presence of excess deoxycytidine) as substrates for dGK ($r = 0.91$, $p < 0.0001$). Leukemic cells isolated from peripheral blood before treatment from B-CLL patients. (D) Comparison of deoxyguanosine and 2-chlorodeoxyadenosine (in presence of excess deoxycytidine) as substrates for dGK ($r = 0.87$, $p < 0.0001$).

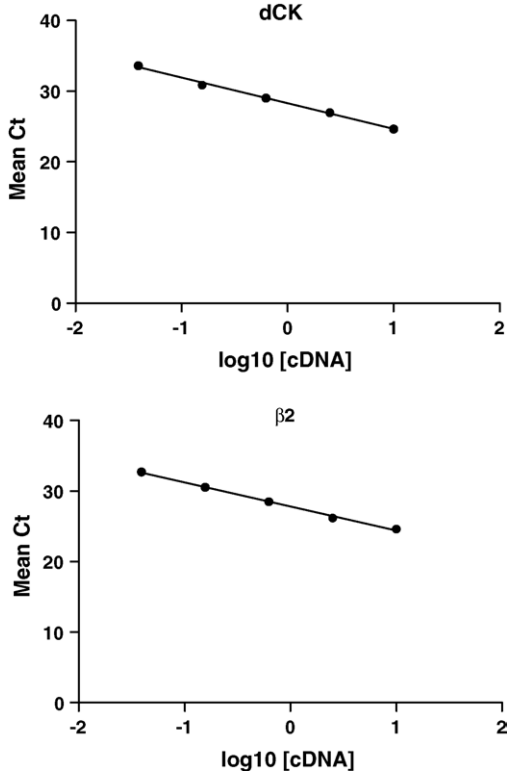

Fig. 3 – Standard curves derived from the amplification plots. RQ-PCR standard concentration between 0.04 and 160 ng RNA dCK and β_2.

The standard curves generated from the amplification plots were linear for both mRNA species ($r \geq 0.965$; Fig. 3). The levels of expression of dCK mRNA were calculated relative to the corresponding level in MOLT-4 cells.

Of the 53 samples subjected to PCR analysis, 8 samples contained degraded RNA or a ratio of 28S to 18S rRNA less than 1.7 and were excluded. The level of expression of dCK mRNA (expressed as the ratio of this level to that of β_2-microglobulin mRNA) in our patients demonstrated extensive inter-individual variability, from 0.011 to 0.189 (Fig. 4). Statistical analysis did not reveal any significant relationship between the levels of dCK mRNAs and the corresponding enzyme activities ($p = 0.35$, $n = 44$; Fig. 5).

3.3. *Silencing of dCK with siRNA*

Exponentially growing CEM cells were assayed for dCK activity. In the siRNA transfected CEM cells we managed to knock-out 62% of the mRNA and 74% of the dCK activity 24 h post-transfection (Fig. 6, upper graph). Both mRNA and activity levels then increased simultaneously and reached the same levels or slightly above as the control cells 96 h after transfection. Activity and expression of dCK was as stable as for the control in the GADPH transfected cells (data not shown). When comparing the dCK mRNA levels and the enzyme activity in the control cells over time we could see a discrepancy between dCK mRNA levels and dCK activity (Fig. 6, lower graph). mRNA levels were kept stable in the electroporated cells while the activity of dCK decreased as a

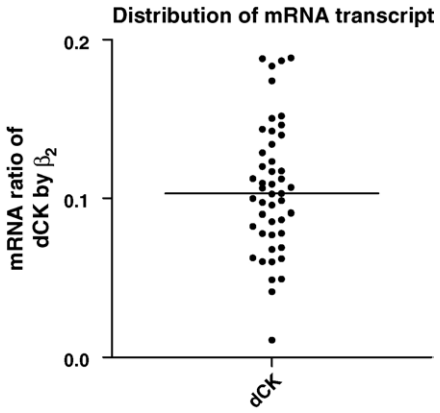

Fig. 4 – Levels of mRNA encoding dCK in peripheral leukocytes from patients with CLL. Total RNA was isolated from peripheral leukocytes obtained from patients newly diagnosed as having B-CLL prior to initiation of chemotherapy ($n = 47$). The levels of dCK mRNA were determined by RQ-PCR and expressed as the ratio of these levels relative to MOLT-4 cells and the mRNA level for the house-keeping gene β_2-microglubulin. The mRNA expression of each gene was determined by calculating the unknown samples relative to a standard of MOLT-4 cells.

consequence of the electroporation procedure itself, suggesting separate regulatory mechanisms for dCK mRNA and activity.

3.4. *RT-PCR analysis of possible mutation in the cDNA for dCK*

Because of the lack of correlation between the levels of dCK mRNA and enzymatic activity, we performed RT-PCR analysis in an attempt to identify the possible presence of the spliced form of dCK as described previously [15]. However, no spliced dCK mRNA could be detected in our samples (Fig. 7), a finding confirmed by the observation of only wild type dCK mRNA in 44 of our samples using mutation analysis of the coding region of this mRNA.

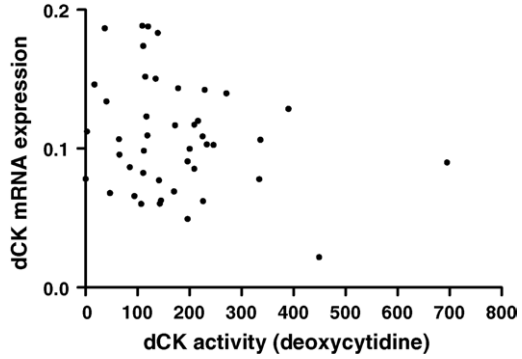

Fig. 5 – Lack of correlation between the levels of dCK mRNA and enzyme activities in peripheral leukocytes from patients with CLL. The relationship between the level of dCK mRNA and activity (with deoxycytidine as substrate) ($p = 0.35$, $n = 44$).

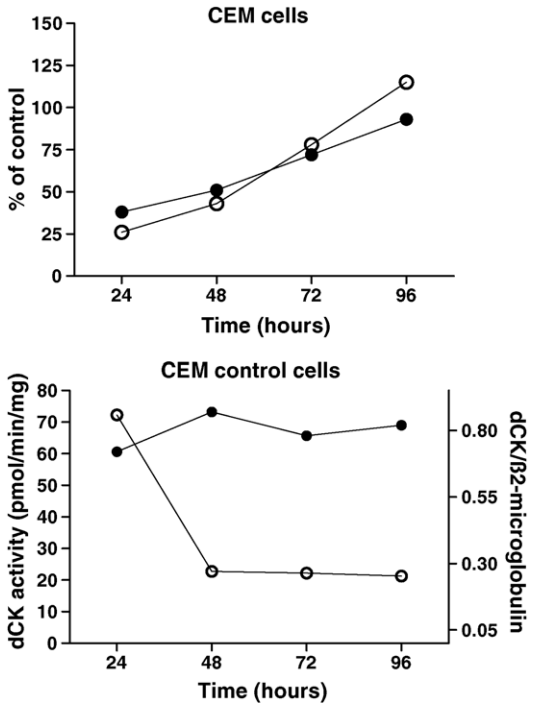

Fig. 6 – Discrepancy between mRNA expression and enzyme activity in electroporated cells lines. CEM cells transfected with siRNA against dCK (upper graph) using electroporation show a simultaneous decrease in both expression and activity of dCK. Control cells (lower graph) electroporated without siRNA show a discrepancy between mRNA expression and protein activity. While the mRNA expression levels remains constant over time, the activity of dCK decreases 24 h after electroporation, suggesting a separate control mechanism for regulating the activity of dCK. (●) dCK mRNA expression and (○) dCK enzyme activity.

4. Discussion

The purine analogues 2-chlorodeoxyadenosine and fludarabine have since the late 1980s come to play a central role in the chemotherapy of B-CLL.

Although cross-resistance to fludarabine and 2-chlorodeoxyadenosine is the rule, certain patients with CLL, who were refractory to fludarabine and still responding to 2-chlorodeoxyadenosine, have been reported, suggesting different mechanisms for resistance to these analogues [17–20], which is also supported by our previous in vitro studies [21,22].

dCK and dGK are the key enzymes phosphorylating a number of important anti-cancer and antiviral drugs. Therefore, it has been assumed that the cytotoxic effects of 2-chlorodeoxyadenosine and fludarabine, on cell culture at least, is determined primarily by the levels at which these enzymes are expressed. Indeed, several studies on in vitro systems have revealed that a decrease in the level of dCK mRNA, protein and/or enzyme activity is associated with increased resistance to nucleoside analogues [22–26]. Further evidence for the importance of dCK in this connection is that transfection of its gene into dCK deficient tumor cell lines

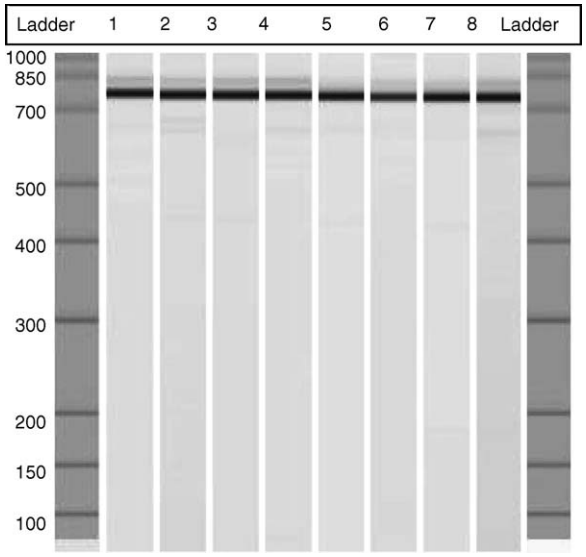

Fig. 7 – No alternatively spliced dCK mRNA could be detected in peripheral leukocytes from patients with CLL. RT-PCR analysis of dCK mRNA was performed on cells from eight patients with CLL prior to initiation of drug treatment. Total 44 samples were analyzed (8 of 44 present here).

restores their sensitivity to cytarabine and 2-chlorodeoxyadenosine [27].

However, very little information concerning cells from patients is presently available. A correlation between refractoriness to nucleoside analogues and low levels or lack of dCK has been reported in patients with acute and chronic leukemia [28–33]. However, other studies indicate no correlation between the outcome of treatment and dCK levels [34–36]. Thus, such activity measurements may be of clinical importance and may contribute to explain the experience from clinical work with CLL that fludarabine exerts its action differently in different groups of patients.

Several studies have suggested that among the methods available, determination of the levels of the mRNA species encoding these enzymes might provide a good indication of clinical sensitivity to nucleoside analogues [33,37–39]. Determination of the levels of dCK and dGK mRNA species has been suggested to provide information on sensitivity. Indeed, we have previously demonstrated that this approach is suitable for the determination of the widely varying levels of dCK and dGK expressed in clinical samples [14]. Cellular resistance due to dCK deficiency can be studied by generating dCK deficient cell lines. In order to create dCK deficient cells without subjecting the cells to any drugs we developed a protocol for transfection of dCK siRNA into CEM cells in an attempt to silence the mRNA and subsequently the dCK enzyme. In these experiments we could see a down-regulation of dCK mRNA and dCK protein activity occurring simultaneously in the cells during a time period of 24–96 h. The siRNA treated samples were correlated to samples treated with GADPH siRNA and cells only subjected to electroporation in order to correct for any non-specific siRNA effects or effects of the electroporation procedure itself. However, the cells treated only with electroporation showed discrepancy between enzyme activity and mRNA expression. The lack of correlation between the activities and levels of mRNA for dCK observed in the present investigation lead us to seriously question the usefulness of this approach. In contrast, in the earlier study, albeit limited number of samples, the enzyme activity and mRNA correlated excellently in human xenografts and solid tumors [39].

Although the mechanism underlying the mRNA expression of dCK without function in the CLL-cells requires further investigation, our clear demonstration here of a lack of correlation between mRNA levels and enzymatic activity suggests post-transcriptional, possibly translational regulation. One possible mechanism is suggested by the structural alteration in the coding region of the dCK gene observed earlier in cell lines and in a small number of samples from patients resistant to cytarabine [40,41]. However, other investigators have detected no such mutations in the dCK gene carried by patients suffering from relapse or in remission [31,32]. A recent report documented a high incidence of alternatively spliced forms of dCK in AML patients resistant to cytarabine [36]. However, transfection of these spliced variants into cell lines was found neither to confer resistance nor to exert any influence on wild type dCK activity [36]. Nonetheless, the mRNA coding for such spliced forms will also be quantitated by PCR if the primers and probes lie outside of spliced regions. However, employing RT-PCR and mutation analysis we could not detect any alternatively spliced or mutated variants of dCK, which might explain the discrepancy between the levels of dCK activity and mRNA in our samples. Indeed, mutations, which inactivate dCK are rarely seen in leukemia patients [41,42].

Although RQ-PCR and gene micro-array are presently the most sensitive procedures for measuring gene expression at the mRNA level, these methods cannot reveal uncharacterized functional mutants or certain forms of post-transcriptional regulation. Lack of correlation between the gene expression and enzymatic activity has been shown before for many different enzymes [35,43–45]. Thus, certain patients with low levels of dCK and dGK enzyme activities may not be identified if mRNA levels only are quantitated, rendering this approach unreliable as a predictive test for sensitivity to treatment with purine and pyrimidine analogues on which to design individualized chemotherapy. In addition, other evidence suggests that factors such as many chemicals, radiation and post-translational modification may change dCK activity without being detectable either by quantization of mRNA or by Western blotting [10,46–48]. Such problems can be overcome by determining phenotype with respect to the enzymatic activities of dCK and dGK towards several substrates as performed in the present investigation. Thus, such activity measurements may be of clinical importance. Further investigations concerning the possible relationship of enzyme activity and/or mRNA levels to clinical response in connection with various hematological malignancies and solid tumors are presently underway in our laboratory.

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