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Expression of Deoxycytidine Kinase and Phosphorylation of 2-Chlorodeoxyadenosine in Human Normal and Tumour Cells and Tissues*

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Deoxycytidine kinase (dCK) activates several clinically important drugs, including the recently developed antileukaemic compound 2-chlorodeoxyadenosine (CdA). The distribution of dCK in cells and tissues has previously been determined by activity measurements, which may be unreliable because of the presence of other enzymes with overlapping substrate specificities. Therefore we have measured dCK polypeptide levels in extracts of normal and malignant human peripheral blood mononuclear cells, gastrointestinal tissues and sarcomas, using a specific immunoblotting technique, as well as the phosphorylation of CdA in the same extracts. High levels of dCK were found in all major subpopulations of normal mononuclear leucocytes (120 ± 19 ng dCK/mg protein) and in B-cell chronic lymphocytic leukaemia (81 ± 30 ng/mg, $n = 23$). Hairy-cell leukaemia contained lower levels (28 ± 23 ng/mg, $n = 7$), as did three samples of T-cell chronic lymphocytic leukaemia (18 ± 14 ng/mg). Phytohaemagglutinin stimulation of normal lymphocytes did not lead to any substantial increase in either dCK activity or protein expression (less than 2.5-fold). The human CEM wt T-lymphoblastoid cell line contained 56 ± 1 ng/dCK/mg protein, while in the CEM ddC50 and AraC8D mutants that lack dCK activity, no dCK polypeptide could be detected. In colon adenocarcinomas, the dCK content was significantly higher (20 ± 9 ng/mg, $n = 20$) than in normal colon mucosa (8 ± 3.5 ng/mg, $n = 19$, $P < 0.05$). A similar pattern of dCK expression was found in gastric adenocarcinomas (21 ± 13 ng/mg, $n = 5$) and normal stomach mucosa (6 ± 5 ng/mg, $n = 5$, $P < 0.15$). One leiomyosarcoma and one extra-skeletal osteosarcoma showed dCK levels comparable with those found in normal lymphocytes (84 ± 6 and 109 ± 4 ng/mg, respectively), while other sarcoma samples contained lower levels, comparable to the gastrointestinal adenocarcinomas (20 ± 7 ng/mg, $n = 12$). Thus, dCK is expressed constitutively and predominantly in lymphoid cells, but it is also found in solid non-lymphoid tissues, with increased levels in malignant cells. The phosphorylation of CdA in crude extracts showed a close correlation to the dCK polypeptide level.

Key words: biochemistry, metabolism, enzymology, drug activation, oncology
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INTRODUCTION

DEOXYCYTIDINE KINASE (EC 2.7.1.74) plays a key role in salvage of deoxyribonucleosides and nucleoside analogues, and has been studied extensively. The enzyme catalyses a 5′-phosphorylation of deoxyribonucleosides using nucleoside triphosphates as phosphate donors [1–4], thereby supplying cells with deoxyribonucleotides for replicative and repair DNA synthesis, as well as for synthesis of liponucleotides [5–7]. The human enzyme was recently cloned [8].

dCK has a broad substrate specificity, including purine deoxyribonucleosides in addition to dCyd [9, 10]. The pharmacological importance of dCK relies on the fact that it is the main enzyme phosphorylating several clinically important cytostatic and antiviral analogues, such as 2′,3′-dideoxycytidine (ddC), 1-β-D-arabinosylcytosine (AraC), 2-chloro-2′-deoxyadenosine (CdA) and 2′-2′-difluorodeoxycytidine (dFdC) [3, 9–13]. Cell lines selected for resistance to these analogues frequently show a lack of dCK activity [14, 15].

It has been shown that dCK is expressed preferentially in lymphoid tissues [1–3], and that high dCyd phosphorylating activity is found in human and murine neoplastic tissues [3, 13, 16–19]. Conflicting results have been published regarding the cell cycle regulation of dCK, with several examples of a clear increase in early S phase [20–23], while others have found that dCK activity is little influenced by cell cycle phases [3, 24, 25].

In earlier studies of dCK, its activity was measured using dCyd as the substrate. This may give rise to several complications: (i) the activities of other enzymes, e.g. cytidine deaminase, deoxycytidylate deaminase and nucleoside phosphorylase, interfere; (ii) substrate kinetics of dCK are complex and show negative cooperativity with several substrates [26]; (iii) there exists at least one other enzyme, TK2, that efficiently phosphorylates dCyd [10, 27], and the presence of TK2 in the extracts leads to an overestimation of the dCK activity. It was suggested [28] that these difficulties can be partially overcome

by using CdA as substrate for dCK, since CdA has been considered to be a substrate solely for dCK, and is resistant to catabolism via adenosine deaminase [29]. However, results were presented that indicated the existence of a second CdA phosphorylating activity [28], and this activity was identified to be dGK, the mitochondrial deoxyguanosine kinase [30]. For these reasons, we wanted to study the expression of dCK in human tissues using an immunological method and correlate the results to CdA phosphorylation in extracts from the same tissues.

CdA is an adenosine deaminase resistant analogue of dAdo [11, 29], which, when given as a drug, mimics the metabolic disturbance and lymphocyte depletion seen in inherited deficiency of adenosine deaminase [31]. The toxicity of CdA to a variety of human haematopoetic cell lines, as well as to resting human peripheral blood mononuclear cells, is well documented (reviewed in [11]). It has also been shown to be toxic to some solid tumours and solid tumour derived cell lines [32–34]. CdA is used in the treatment of patients with chronic lymphocytic leukaemia, low-grade lymphomas and most successfully hairy-cell leukaemia, with a good patient tolerance and minimal non-haematopoetic side effects (reviewed in [35]). If other human malignancies display high phosphorylation of CdA, they might be new potential targets for CdA therapy.

The availability in this laboratory of pure human dCK has enabled the production and purification of antibodies against the enzyme [27]. Here we describe the development of a specific and quantitative immunoblotting method for human dCK in crude cell and tissue extracts.

MATERIALS AND METHODS

Materials

[5-^{3}H]-dCyd (22 Ci/mmole), [methyl-^{3}H]-Thd (25 Ci/mmole), [^{125}I]-Protein A and Hyperfilm MP were purchased from Amersham (U.K.), while [8-^{3}H]-CdA (20 Ci/mmole) and 3′-azido-[methyl-^{3}H]-Thd ([^{3}H]-AZT, 20 Ci/mmole) came from Moravek Biochemicals (La Brea, California, U.S.A.). Unlabelled deoxyribonucleosides were obtained from Sigma (St. Louis, Missouri, U.S.A.). Discs used in the enzymatic assays were Whatman DE-81 filter paper discs. Membranes used for immunoblotting were ProBlott membrane (Applied Biosystems, Foster City, California, U.S.A.) or PVDF membrane (0.2 micron, Bio Rad Lab, Richmond, California, U.S.A.). All other chemicals, materials and reagents were of the highest quality commercially available.

Patient samples

Peripheral blood mononuclear cells were isolated from patients with chronic lymphocytic leukaemia of B-cell and T-cell types and hairy-cell leukaemia using Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. The cells were characterised at pathology departments of local hospitals, with B- and T-cell subgrouping using panels of monoclonal antibodies. The chronic lymphocytic leukaemia (CLL) samples contained > 90% leukaemic cells, whereas in hairy-cell leukaemia (HCL) the fraction of leukaemic cells was variable. In all samples > 95% of the cells were resting in G_0/G_1, as determined by flow cytometry. Frozen cell pellets were stored at −70°C until extracted as described below. Samples of solid normal and tumour tissues were taken during surgery, immediately frozen in liquid nitrogen and then stored at −70°C until protein extraction. The diagnoses and histological characteristics of the solid tumours were obtained from the Department of Tumour Pathology at the Karolinska Hospital. For gastrointestinal adenocarcinomas, mucosa of the uninvolved and histologically normal colon and ventricle, without any part of the muscle layers, was taken as control tissue. The non-gastrointestinal tumours consisted of a variety of different soft-tissue sarcomas (crudely characterised as four fibrosarcomas, three soft part osteosarcomas, two alveolar soft part sarcomas, one each of epitheloid cell sarcoma, leiomyosarcoma, histiocytoma, neurofibrosarcoma and pleomorph sarcoma). Two are described in more detail in the Results and Discussion sections.

Cell cultures

CCRF-CEM cells, a human malignant T-lymphoblastoid cell line, as well as the mutants CEM ddC50 and CEM AraC8D (both dCK$^-$, 14, 15), CEM 5-bromo-2′-deoxyuridine (BrdUrd) (TK 1$^-$) and CEM AG 1 hypoxantine-guanine phosphoribosyl transferase negative (HGPRT$^-$) were kindly provided by B. Ullman (Department of Biochemistry and Molecular Biology, University of Oregon, Portland, Oregon, U.S.A.). Cells were grown at 37°C in Dulbecco's modified medium containing 10% heat-inactivated (56°C, 30 min) horse serum, in a humidified 7% CO_2 atmosphere.

Normal peripheral blood mononuclear cells

Buffy-coat leucocytes were obtained from healthy donors (Karolinska Hospital, Stockholm, Sweden), from which peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Monocytes were isolated by plastic adherence for 60 min at 37°C, and after removal of non-attached peripheral blood lymphocytes (PBL), were released by EDTA treatment (15 mM) at 0°C for 90 min. The PBL were passed through a nylon wool column for separation of B- and T-lymphocytes [36]. The effluent cells were further separated for T- and null cells by precipitation of the T-cells by sheep red blood cells (SRBC) rosetting. The B-cells eluted from the nylon wool column were also further purified by one extra SRBC rosetting. These routine procedures have been previously described in more detail [36, 37], yielding a T-cell fraction with > 80% T-cells and a B-cell fraction with > 85% B-cells. Mitogen stimulation of PBL or separated T-cells (2×10^6 cells/ml in RPMI medium containing 7.5% fetal calf serum (FCS) was performed by incubation with phytohaemagglutinin A (PHA) (1 μg/ml) for 72 h at 37°C.

Cell and tissue extraction

Frozen cell pellets were suspended in extraction buffer (30–60 $\times 10^6$ cells/ml) containing 50 mM TRIS-HCl pH 7.6, 2 mM dithiothreitol (DTT), 5 mM benzamidine, 0.5 mM phenylmethyl-sulfonyl-fluoride (PMSF), 20% glycerol and 0.5% Nonidet P40, whereupon they were freeze–thawed three times for

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disruption of cell membranes and organellae. Frozen solid tissue samples were thawed and homogenised in the same extraction buffer using a DISP 25 homogeniser (Intermed). The extracts were then centrifuged for 20 min at 12 000 rpm after which supernatants containing the cellular proteins were kept at −70°C until analysed. Protein concentrations in the extracts were measured using the Biorad protein assay, with bovine serum albumin (BSA) as a standard.

Enzyme assays

Deoxycytidine kinase activity was assayed with [5-^{3}H]-dCyd (10 μM) or [8-^{3}H]-CdA (50 μM), thymidine kinase with [methyl-^{3}H]-AZT (20 μM) as substrates in a reaction mixture containing 50 mM TRIS-HCl pH 7.6, 5 mM $MgCl_2$, 5 mM ATP, 2 mM DTT, 10 mM sodium fluoride and 0.2–0.5 mg/ml of cell extract. Specific activities of the labelled compounds were in all cases previously 1000 cpm/pmole. The enzyme assays were carried out at 37°C, and, after appropriate time intervals to yield a linear reaction rate, 10 μl aliquots were spotted on DEAE cellulose filters, which were then washed, eluted and counted as described previously [28].

Antibodies to dCK

Immune serum against pure human dCK was produced and affinity purified as described previously [27]. The concentration of purified antibody was approximately 20 μg/ml, and the working dilution used in immunoblots was 1 : 25 in TRIS-buffered saline (TBS, i.e. 25 mM TRIS-HCl pH 7.6 and 150 mM NaCl) containing 3% BSA and 0.02% NaN_3.

Immunoblotting

Protein from cell extracts or purified dCK was precipitated with 5% TCA and dissolved in 400 mM TRIS-base containing 25% glycerol, 1% SDS, 0.01% bromophenol blue and 10 mM DTT. Samples were incubated for 10 min at room temperature, neutralised and boiled for 2 min before they were applied to a 12% polyacrylamide slab gel. The electrophoresis was performed as described previously [27]. Electrophoretic transfer to ProBlott or PVDF membranes (Biorad) was carried out at 225 mA for 1.5 h using the Mini Trans-Blot Electrophoretic Transfer Cell (Biorad) as described by the supplier. These conditions permitted total transfer of dCK to ProBlott or PVDF sheets, with no protein penetrating through the membrane (usual nitrocellulose membranes resulted in leakage of dCK through the membranes). The sheets were allowed to dry, then rinsed three times with TBS and blocked for 2 h at room temperature with 3% BSA in TBST (TBS with 0.1% (v/v) Tween-R). After blocking, the sheets were rinsed with TBS and soaked with gentle agitation for 16 h in the dCK antibody working solution. Sheets were then washed with TBST (two times for 5 min, two to three times for 10 min) and incubated with gentle agitation for 2 h at room temperature in a solution of 0.3 μCi/ml [^{125}I]-Protein A in TBS with 3% BSA and 0.02% NaN_3. After this, they were washed several times in TBST, rinsed in TBS, blotted dry and autoradiographed with Hyperfilm MP at −70°C for 6 h to 3 days (control experiments confirmed that film blackening was linear with the amount of protein and the developing times used in this study). The blackening of the films were measured using a double-beam recording microdensitometer (Joyce, Loeble and Co. Ltd, U.K.).

Quantitation of dCK in immunoblotting

Control amounts (1.25–10 ng of electrophoretically pure dCK) from leukaemic spleen purified as described previously [38] as well as partially purified dCK (from the hydroxylpatite step, 0.25–1.5 μg) were electrophoresed, immunoblotted, autoradiographed and scanned as described above. The fraction of dCK polypeptide in the partially purified dCK was 5 ± 1 ng dCK/μg protein, determined by comparison with preparations of pure dCK. This partially purified dCK was then used as an internal standard in all subsequent immunoblots.

Statistical analysis

Statistics analysis was performed using the Statview 512+™ version 1.0 computer programme from Abacus Concepts, Berkeley, California, U.S.A. The Fisher's PLSD test with a significant F-test was used to determine the significance differences of dCK levels between the sample groups. The linear regression feature was utilised to determine the overall correlation between dCK content and CdA phosphorylation.

RESULTS

To determine the specificity of the anti-dCK antibody, we used extracts of mutant human T-lymphoma cell lines, CEM-ddC50 and CEM-araC8D, which have lost dCK activity upon selection for resistance against the analogues ddC and AraC [14, 15]. In extracts of neither cell line (Figure 1) nor in extracts of normal human brain tissue (not shown), that also lacks dCK activity [28, 30], could any dCK polypeptide be detected. In contrast, CEM wt and other CEM mutants, resistant to BrdUrd or 6-thioguanine (due to lost activities of thymidine kinase or HGPRT, respectively) contained significant and comparable levels of dCK (Figure 1). These results demonstrate that the immunoblotting procedure was specific for detection and quantitation of the dCK polypeptide.

Some leukaemic cells, tumours and normal tissues contained proteins with a high molecular weight, most likely immunoglobulins, which bound [^{125}I]-Protein A and thereby interfered with the quantitation of dCK polypeptide. A higher concentration of DTT (10 mM) in the sample buffer during the denaturation step prior to electrophoresis not only abolished the binding of Protein A to the high molecular weight proteins, but also resulted in better detection of the 30 kDa dCK band (not shown). This was a methodological improvement which enabled the use of the immunoblotting procedure with crude extracts from the different clinical samples.

The dCK content varied very little between extracts of the major subpopulations of human normal peripheral blood mononuclear cells, either when calculated per mg of protein (91–145 ng dCK/mg, Table 1) or per cell (3.5–9.8 ng dCK/10^6 cells). Although the dCK level in mature T-lymphocytes when calculated per mg of protein (Table 1) was twice as high as that found in the T-lymphoblastoid CEM wt cell line (56 ± 1 ng dCK/mg), it was similar when calculated per cell (4.2 ± 0.6 ng/10^6 cells in T-lymphocytes and 5.2 ± 0.1 ng/10^6 cells in CEM wt). Neither in PBMC nor in T-lymphocytes did dCK increase more than 2.5-fold upon mitogen stimulation. As expected, TK1 activity, which was assayed by phosphorylation of 3'-azidothymidine (AZT) [10, 28], increased more than 100-fold in PHA stimulated PBMC and 200-fold in stimulated T-lymphocytes (Table 1).

The dCK protein levels and CdA phosphorylation in extracts of peripheral blood mononuclear cells obtained from 33 patients with different types of leukaemias were also analysed (Table 1). In B-CLL a 5-fold variation in dCK content was observed but the majority of the samples contained 50–110 ng dCK per mg of cell protein. In HCL dCK content per protein was significantly

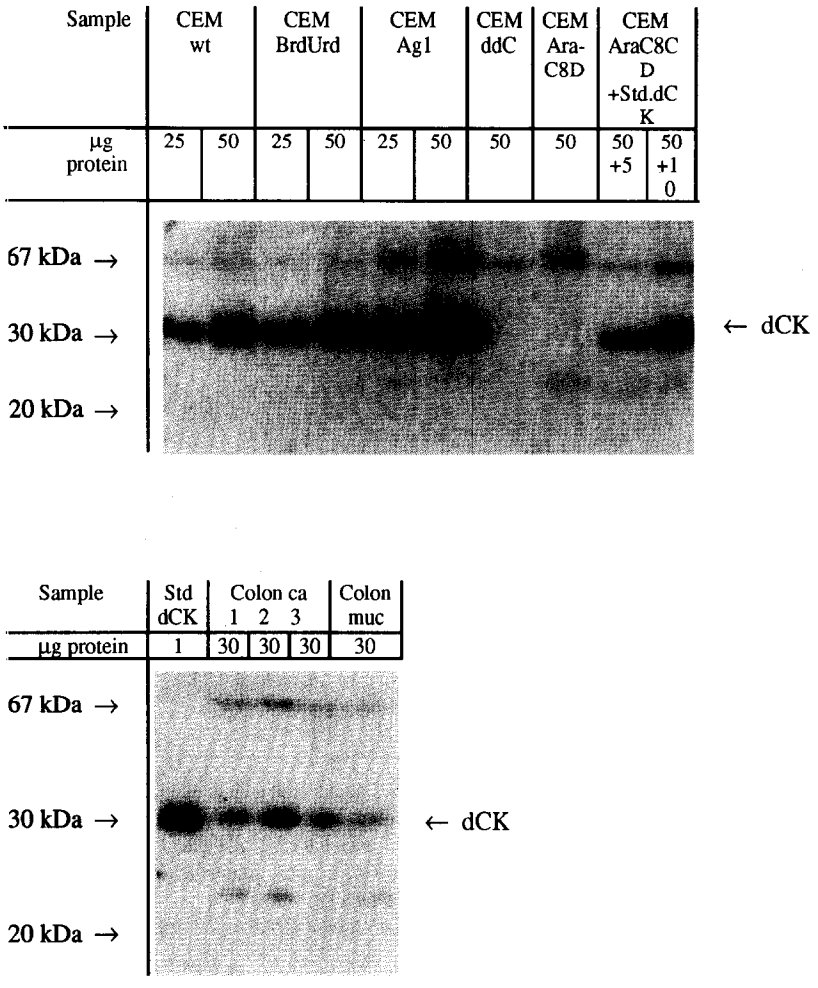

Figure 1. Western blotting analysis of the expression of dCK in CEM wt and mutant CEM cell lines as well as normal and tumour samples. The lanes represent, as indicated in the figure, protein from extracts of CEM wt, CEM BrdUrd (TK^-), CEM AG 1 ($HGPRT^-$), CEM ddC50 (dCK^-) and CEM AraC8D (dCK^-), and as a standard, partially purified dCK electrophoresed together with the extract of CEM AraC8D cells. The lower part of the figure shows extracts from two different colon adenocarcinomas and one normal colon mucosa as well as a sample of partially purified standard dCK.

lower than in B-CLL (28 ± 24 ng/mg, $P < 0.001$). Three T-CLL samples were analysed and, in spite of the small number of cases, they also displayed significantly lower levels of dCK than that found in B-CLL (18 ± 14 ng/mg, $P < 0.01$). CdA phosphorylation and dCK polypeptide expression in these leukaemia samples showed a very good correlation (Table 1).

dCK levels and CdA phosphorylation in selected solid tissues were also determined (Table 1). The level of dCK polypeptide was low in normal colon mucosa (8 ± 3.5 ng/mg) and normal stomach mucosa (6 ± 5 ng/mg) and dCK could not be detected in extracts from normal muscle. Higher dCK levels were found in the gastrointestinal adenocarcinomas, although the difference from corresponding normal mucosa was more significant in the colon cancer samples (20 ± 9 ng dCK/mg protein, $P < 0.05$) than in the smaller number of stomach cancer samples (21 ± 13 ng dCK/mg, $P < 0.15$). Nineteen cases of colon and three cases of stomach cancer were available for comparison of dCK expression between normal and malignant tissue from the same patient. dCK increased more than 4-fold compared with the normal sample in four of the colon cancer samples (21%); in nine cases (47%) the dCK level was 2–3 times higher and in six cases (32%) it was less than 2 times higher in tumour than in normal tissue. Three gastric adenocarcinomas with available control tissue from the same patient showed 3- and 16-fold increases of dCK content in two of the samples but no difference between the normal and malignant tissue in the third sample.

The mean dCK content in extracts from twelve soft tissue sarcomas was similar to the level of dCK observed in the gastrointestinal adenocarcinomas (Table 1). In addition to this, two soft-tissue sarcomas showed unexpectedly high dCK levels (84 ± 6 and 109 ± 4 ng/mg protein, respectively). One was a liver metastasis of a leiomyosarcoma, and the other was an extra-skeletal osteosarcoma removed from the triceps muscle of a patient. Neither of the samples had high lymphocyte infiltration and the surrounding normal tissues, liver and muscle, had undetectable dCK levels [26–28, Table 1]. The high dCK levels in these two sarcomas must therefore be derived from the tumour cells, and not from the surrounding tissue. It is important to note that among the twelve sarcomas with lower dCK levels, other extra-skeletal osteosarcomas were included, indicating a large variation in dCK levels between sarcomas of the same classification.

Western blotting results with extracts from normal muscle and colon mucosa, as well as from colon carcinoma tumours showed, in addition to the 30 kDa dCK polypeptide a major cross-reacting band of 29 kDa in muscle extracts and a minor

Table 1. Expression of deoxycytidine kinase in human cells and tissues

	CdA* pmoles per min/mg	ng Ag† per mg		dCyd* (pmoles per min/mg)	AZT‡
Normal leucocytes					
PBMC	281 ± 8	91 ± 10		70 ± 1	3
PBMC + PHA	395 ± 30	132 ± 24		88 ± 4	370
Monocytes	363 ± 29	106 ± 7		80 ± 1	12
T-lymphocytes	414 ± 3	113 ± 16		99 ± 9	2
T+PHA	345 ± 21	114 ± 14		63 ± 3	427
B-lymphocytes	497 ± 115	137 ± 20		198 ± 6	<1
Null cells	443 ± 12	145 ± 27		112 ± 3	4
PBMC of leukaemia patients					
B-CLL	340 ± 90	81 ± 30	(23)		
T-CLL	95 ± 13	18 ± 14	(3)		
HCL	160 ± 110	28 ± 23	(7)		
Normal solid tissue					
Gastric mucosa	38 ± 12	6 ± 5	(5)		
Colon mucosa	50 ± 25	8 ± 3.5	(19)		
Muscle	n.d.	<0.5	(2)		
Solid-cancer tissue					
Gastric adenocarcinoma	105 ± 35	21 ± 13	(5)		
Colon adenocarcinoma	90 ± 50	20 ± 9	(20)		
Soft tissue sarcoma	78 ± 41	20 ± 7	(12)		
→ One leiomyosarcoma	190 ± 10	84 ± 6			
→ One osteosarcoma	240 ± 12	109 ± 4			

*dCK activity was determined using CdA or dCyd as substrates as described in Materials and Methods and values are pmoles of product formed per min and mg of protein with the standard deviations. The values in parentheses are the number of cases analysed.
†The amount of dCK antigen was determined with immunoblotting and the purified dCK antibody as described in Materials and Methods.
‡TK1 activity was determined using AZT as the substrate as described in Materials and Methods.
n.d. = not determined.

band of 22 kDa in extracts from colon carcinomas (Figure 1). When an excess of purified dCK was added to the antibody solution, immunostaining of the 22 kDa band was drastically reduced but it was unchanged for the 29 kDa protein (data not shown). This competition experiment demonstrated that there are, in some cases, unrelated proteins that may cross-react with the antibody but overall this immunoblotting method is a very sensitive tool to study dCK expression in cell extracts. The 22 kDa band is most likely to be a degradation product of the 30 kDa dCK protein and it was observed in four of the 20 colon carcinoma extracts tested here. We did not detect the 22 kDa band in any other extract but in a recent investigation of a series of cultured human ovarian cell lines, selected for resistance to dFdC, a dCK band with similar molecular weight was observed using the same antibody and this band was interpreted as a proteolysed form of the enzyme [39].

When we measured the CdA phosphorylation in all the samples, a good correlation ($r = 0.883$) to the dCK content was found (Figure 2) and an apparent specific activity of 3.1 μmole CdA phosphorylated per min per mg dCK. This was a better correlation than that found between the dCK content and phosphorylation of dCyd ($r = 0.554$), which was measured in the normal blood cells, in the majority of the B-CLL and in eight of the colon samples. The apparent specific activity in this case was 0.66 μmole dCyd phosphorylated per min per mg dCK. Moreover, the apparent specific activity of dCK in extracts of

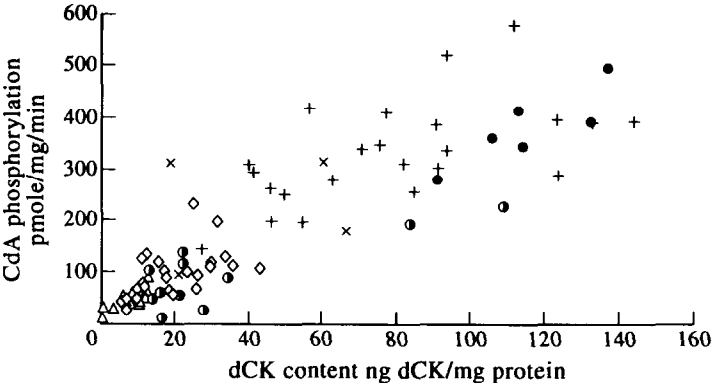

Figure 2. The level of dCK determined by immunoblotting and the phosphorylation of CdA in normal and malignant human cells and tissues. CdA phosphorylation in extracts of normal PBMC (●), B-CLL cells (+), HCL cells (×), normal gastrointestinal mucosa (△), gastrointestinal adenocarcinomas (◇) and soft tissue sarcomas (◑) were plotted against the dCK content in the same extracts, as determined by immunoblotting. An overall regression analysis showed an apparent specific activity of 3.1 μmole CdAMP/mg dCK/min with $r = 0.883$.

normal blood cells showed a small variation in CdA phosphorylation (3.0–3.7 μmole CdAMP per min per mg dCK), whereas the variation was larger when dCyd was used as the substrate (0.55–1.4 μmole dCMP per min per mg dCK).

DISCUSSION

Several studies have shown that cultured T-lymphoblastoid cell lines have higher dCK levels than B-lymphoblastoid cell lines (reviewed in [40]). The results presented here show that normal mature B- and T-lymphocytes as well as monocytes and null cells express high and similar levels of dCK polypeptide and dCK activity. The reason for the discrepancy between lymphoblastoid cell lines and activated peripheral B- and T-cells is not clear but is probably related to differences in dCK expression in immature compared with differentiated lymphocytes. As stated in the Introduction, conflicting results have been published regarding the cell cycle regulation of dCK. Here we observed a minor increase in dCK polypeptide (maximally 2–3 fold) upon mitogen stimulation of lymphocytes. However, the level of dCK in resting lymphocytes was approximately five times higher than that found in other tissues. Thus, the variability between various cell types in dCK expression is much larger than that between cell cycle phases.

Results by Mitchell and colleagues [41] support this conclusion since they found no increase of dCK mRNA levels in peripheral blood T-lymphocytes after mitogen stimulation. A recent study of dCK expression in four different cell lines demonstrated no cell cycle variation of dCK mRNA levels in any case but increased dCK activity in S phase cells in two cell lines but not in the other two [42]. Taken together these results indicate that dCK expression is regulated in a tissue or cell type specific manner both at the transcriptional and at the post-transcriptional level.

The CEM mutants ddC50 and AraC8D have been studied extensively in order to determine the mechanism for their loss of dCK activity [8, 14, 15]. Chottiner and associates have shown decreased levels of dCK mRNA in both of these cell lines [8] and recently Owens and coworkers showed that two mutations in the dCK gene of AraC-8D resulted in a loss of catalytic activity combined with increased proteolysis of the enzyme [43]. A point mutation in one allele in the ddC50 cell line was identified that resulted in a reduced catalytic activity and the authors postulated that the second allele was not expressed [43]. The results presented here show that neither AraC-8D nor ddC50 contain detectable levels of the dCK polypeptide, indicating that the mRNA in neither one of these mutants is translated, or that the proteolysis of mutant dCK is as high in ddC50, as it is in AraC8D [43]. Kawasaki and coworkers did not detect any dCK polypeptide in two other dCK negative mutants [44]. We observed here an apparent 22 kDa intermediate in the proteolysis of dCK in some colon carcinoma extracts but not in the extracts from other tissues, and thus there is probably a tissue specific degradation process that may be important in regulating the levels of this enzyme.

In malignant lymphoid cells containing dCK, Kawasaki and coworkers [44] reported dCK levels 100 times higher than those determined here. This discrepancy is probably due to the different standard dCK preparations used (i.e. recombinant fusion protein compared with purified spleen enzyme). The calculated apparent specific activity of dCK in this study is in the same range as that for highly purified dCK using dCyd as the substrate [38], and approximately 100-fold higher than that reported in the other study [44]. Nevertheless, Kawasaki and colleagues showed that the activity of dCK, using dCyd as the substrate, correlated well with the amount of dCK in different samples [44]. This is supported by our data, although we found a better correlation between dCK content and CdA phosphorylation than that between dCK content and dCyd phosphorylation. Therefore, CdA could preferentialy be used instead of dCyd as the substrate in dCK activity measurements [28].

A question of clinical interest is whether or not the level of dCK in leukaemic cells may influence the response to CdA therapy. Kawasaki and coworkers have presented evidence that this is the case [44]. However, the phosphorylation of CdA is clearly not the only factor responsible for its cytotoxic and therapeutic effects, e.g. 5′-nucleotidase, has been proposed to play an important role in inhibiting the chemotherapeutic efficacy of CdA [45]. In a study using different cell lines, it was shown that a 100-fold range in cell sensitivity to CdA could not be explained by different levels of CdA nucleotides [33]. The exact mechanism of action of CdA is not known. It seems to be different in dividing and quiescent cells, and includes inhibition of ribonucleotide reductase, stimulation of apoptosis, accumulation of DNA strand breaks and NAD^+ and ATP depletion [11, 35, 45]. In the present study, we show that in HCL, where the best response to CdA treatment is seen, the dCK level with its accompanying CdA phosphorylation is lower than in B-CLL, a leukaemia that displays a variable response to treatment. Therefore, our results, as well as those of others, indicate that differences at the target(s) for CdA action are probably more important than differences in the rate of anabolic phosphorylation of the drug, at least in certain cell types.

The toxicity of CdA to both proliferating and resting cells could conceivably be a unique advantage in treatment of slow growing solid tumours. Previous *in vitro* data demonstrated that CdA is cytotoxic to cells of certain solid tumours [32] and to some neuroblastoma [33] and melanoma [34] cell lines, at varying inhibitory concentrations. It was not clear to what extent these differences in toxic concentration of CdA reflected differences in anabolic phosphorylation or differences at the target level. This study shows that dCK expression is higher in a majority of gastrointestinal adenocarcinoma samples than in corresponding normal mucosa, but the average dCK content in these adenocarcinomas, as well as in 12 samples of sarcomas, was approximately 5-fold lower than in normal or B-CLL lymphoid cells. Whether increased dCK levels in certain tumours can serve as the basis for improved CdA efficacy remains to be determined. A recent study by Ruiz van Harperen and associates [13] in human tumour xenografts showed a large variation in dCK levels with no correlation to sensitivity for the toxicity of cytidine analogues. They tested four sarcoma tumour extracts and none had high dCK activity. The two cases of soft-tissue sarcomas with high dCK levels reported here are, however, of special interest since this result shows that solid tumours may display levels of dCK and CdA phosphorylation comparable with those of normal and malignant lymphocytes, cells in which CdA exerts its well documented therapeutic effect.

1. Sugino Y, Teraoka H, Shimono H. Metabolism of deoxyribonucleotides. I. Purification and properties of deoxycytidine monophosphokinase of calf thymus. *J Biol Chem* 1966, **241**, 961–969.
2. Momparler RL, Fischer GA. Mammalian deoxynucleoside kinase. I. Deoxycytidine kinase: purification, properties, and kinetic studies with cytosine arabinoside. *J Biol Chem* 1968, **243**, 4298–4304.
3. Durham JP, Ives DH. Deoxycytidine kinase. I. Distribution in normal and neoplastic tissues and interrelationships of deoxycytidine and 1-beta-D-arabinofuranosylcytosine phosphorylation. *Mol Pharmacol* 1969, **5**, 358–375.
4. Durham JP, Ives DH. Deoxycytidine kinase. II. Purification and general properties of the calf thymus enzyme. *J Biol Chem* 1970, **245**, 2276–2284.

5. Medrano E, Iujvidin S, Mordoh J. Synthesis of phosphatidyl-dCMP in human lymphocytes and its enhancement by chlorpromazine. *Eur J Biochem* 1981, **116**, 249–253.
6. Spyrou G, Reichard P. Compartmentation of dCTP pools. Evidence from deoxyliponucleotide synthesis. *J Biol Chem* 1987, **262**, 16425–16432.
7. Spasokukotskaja T, Taljanidisz M, Sasvári-Székely M, Staub M. Deoxycytidine is salvaged not only into DNA but also into phospholipid precursors. III. dCDP-diacylglycerol formation in tonsillar lymphocytes. *Biochem Biophys Res Commun* 1991, **174**, 680–687.
8. Chottiner EG, Shewach DS, Datta NS, *et al.* Cloning and expression of human deoxycytidine kinase cDNA. *Proc Natl Acad Sci USA* 1991, **88**, 1531–1535.
9. Krenitsky TA, Tuttle JV, Koszalka GW, *et al.* Deoxycytidine kinase from calf thymus. Substrate and inhibitor specificity. *J Biol Chem* 1976, **251**, 4055–4061.
10. Eriksson S, Kierdaszuk B, Munch-Petersen B, Öberg B, Johansson NG. Comparison of the substrate specificities of human thymidine kinase 1 and 2 and deoxycytidine kinase toward antiviral and cytostatic nucleoside analogs. *Biochem Biophys Res Commun* 1991, **176**, 586–592.
11. Plunkett W, Saunders PP. Metabolism and action of purine nucleoside analogs. *Pharmacol Ther* 1991, **49**, 239–268.
12. Ghandi V, Plunkett W. Modulatory activity of 2′,2′-difluorodeoxycytidine on the phosphorylation and cytotoxicity of arabinosyl nucleosides. *Cancer Res* 1990, **50**, 3675–3680.
13. Ruiz van Haperen VWT, Veerman G, Braakhuis BJM, *et al.* Deoxycytidine kinase and deoxycytidine deaminase activities in human tumor xenografts. *Eur J Cancer* 1993, **29A**, 2132–2137.
14. Ullman B, Coons T, Rockwell S, McCartan K. Genetic analysis of 2′,3′-dideoxycytidine incorporation into cultured human T-lymphoblasts. *J Biol Chem* 1988, **263**, 12391–12396.
15. Hershfield MS, Fetter JE, Small WC, *et al.* Effects of mutational loss of adenosine kinase and deoxycytidine kinase on deoxyATP accumulation and deoxyadenosine toxicity in cultured CEM human T-lymphoblastoid cells. *J Biol Chem* 1982, **257**, 6380–6386.
16. Harkrader RJ, Jackson RC, Ross DA, Weber G. Increase in liver and kidney deoxycytidine kinase activity linked to neoplastic transformation. *Biochem Biophys Res Commun* 1980, **96**, 1633–1639.
17. Denton JE, Lui MS, Aoki T, *et al.* Enzymology of pyrimidine and carbohydrate metabolism in human colon carcinomas. *J Cancer Res* 1982, **42**, 1176–1183.
18. Madani S, Baillon J, Fries J, *et al.* Pyrimidine pathways enzymes in human tumors of brain and associated tissues: potentialities for the therapeutic use of *N*-(Phosphonacetyl-L-aspartate and 1-β-D-arabinofuranosylcytosine. *Eur J Cancer Clin Oncol* 1987, **23**, 1485–1490.
19. Braakhuis BJM, Van Dongen GAMS, Peters GJ, Van Walsum M, Leyva A, Snow GB. Chemotherapy of human head and neck cancer xenografts with 5-aza-2′-deoxycytidine. In RL Momparler, D de Vos, eds. *5-Aza-2′-deoxycytidine: Preclinical and Clinical Studies*. Haarlem, PCH, 1990, 67–76.
20. Persohn LK, Drewinko B, Loo TL. Uptake of 1-beta-D-arabinofuranosyl cytosine and prednisolone by synchronized lymphoma cells. *Med Pediat Oncol* 1976, **2**, 291–295
21. Pegoraro L, Bernengo MG. Thymidine kinase, deoxycytidine kinase and deoxycytidylate deaminase activities in phytohaemagglutinin stimulated human lymphocytes. *Exp Cell Res* 1971, **68**, 283–290.
22. Brent TP. Periodicity of DNA synthetic enzymes during the HeLa cell cycle. *Cell Tissue Kinet* 1971, **4**, 297–305.
23. Wan CW, Mak TW. Kinase and deaminase in synchronized NRK cells. *Cancer Res* 1978, **38**, 2768–2772.
24. Richel DJ, Colly LP, Arentsen-Honders MW, Starrenburg CWJ, Wilemze R. Deoxycytidine kinase, thymidine kinase and cytidine deaminase and the formation of ara-CTP in leukemic cells in different phases of the cell cycle. *Leuk Res* 1990, **14**, 363–369.
25. Arnér ESJ, Flygar M, Bohman C, Wallström B, Eriksson S. Deoxycytidine kinase is constitutively expressed in human lymphocytes: consequences for compartmentation effects, unscheduled DNA synthesis, and viral replication in resting cells. *Exp Cell Res* 1988, **178**, 335–342.
26. Kierdaszuk B, Eriksson S. Selective inactivation of the deoxyadenosine phosphorylating activity of pure human deoxycytidine kinase: stabilization of different forms of the enzyme by substrates and biological detergents. *Biochemistry* 1990, **29**, 4109–4114.
27. Habteyesus A, Nordenskjöld A, Bohman C, Eriksson S. Deoxynucleoside phosphorylating enzymes in monkey and human tissues show great similarities, while mouse deoxycytidine kinase has a different substrate specificity. *Biochem Pharmacol* 1991, **42**, 1829–1836.
28. Arnér ESJ, Spasokoukotskaja T, Eriksson S. Selective assays for thymidine kinase 1 and 2 and deoxycytidine kinase and their activities in extracts from human cells and tissues. *Biochem Biophys Res Commun* 1992, **188**, 712–718.
29. Bennett LL Jr, Chang C-H, Allan PW, *et al.* Metabolism and metabolic effects of halopurine nucleosides in tumor cells in culture. *Nucleosides & Nucleotides* 1985, **4**, 107–116.
30. Wang L, Karlsson A, Arnér ESJ, Eriksson S. Substrate specificity of mitochondrial 2′-deoxyguanosine kinase: efficient phosphorylation of 2-chlorodeoxyadenosine (CdA). *J Biol Chem* 1993, **268**, 22847–22852.
31. Giblett ER, Anderson JE, Cohen F, Pollara B, Meuwissen HJ. Adenosine-deaminase deficiency in two patients with severely impaired cellular immunity. *Lancet* 1972, **2**, 1067–1069.
32. Hutton JJ, Von Hoff DD. Cytotoxicity of 2-chlorodeoxyadenosine in a human tumor colony-forming assay. *Cancer Drug Deliv* 1986, **3**, 115–122.
33. Avery TL, Rehg JE, Lumm WC, Harwood FC, Santana VM, Blakley RL. Biochemical pharmacology of 2-chlorodeoxyadenosine in malignant human hematopoietic cell lines and therapeutic effects of 2-bromodeoxyadenosine in drug combinations in mice. *Cancer Res* 1989, **49**, 4972–4978.
34. Parsons PG, Bowman EPW, Blakley RL. Selective toxicity of deoxyadenosine analogues in human melanoma cell lines. *Biochem Pharmacol* 1986, **35**, 4025–4029.
35. Beutler E. Cladribine (2-chlorodeoxyadenosine). *Lancet* 1991, **340**, 952–956.
36. Julius MH, Simpson E, Herzenberg LA. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur J Immunol* 1973, **3**, 645–649.
37. Masucci MG, Bejarano MT, Masucci G, Klein E. Large granular lymphocytes inhibit the *in vitro* growth of autologous Epstein-Barr virus-infected B cells. *Cell Immunol* 1983, **76**, 311–321.
38. Bohman C, Eriksson S. Deoxycytidine kinase from human leukemic spleen: preparation and characteristics of homogeneous enzyme. *Biochemistry* 1988, **27**, 4258–4265.
39. Ruiz van Harperen VWT, Veerman G, Eriksson S, *et al.* Development and molecular characterization of a 2′,2′-difluorodeoxycytidine-resistant variant of the human ovarian carcinoma cell line A2780. *Cancer Res* 1994, **54**, 4138–4143.
40. Martin DJ, Gelfand EW. Biochemistry of diseases of immunodevelopment. *Ann Rev Biochem* 1981, **50**, 845–877.
41. Mitchell BS, Song JJ, Johnsson EE, Chen E, Dayton JS. Regulation of human deoxycytidine kinase expression. *Adv Enzyme Reg* 1993, **33**, 61–70.
42. Hengstschläger M, Denk C, Wawra E. Cell cycle regulation of deoxycytidine kinase. Evidence for post-transcriptional control. *FEBS Lett* 1993, **321**, 237–240.
43. Owens JK, Shewach DS, Ullman B, Mitchell BS. Resistance to 1-beta-D-arabino-furanosylcytosine in human T-lymphoblasts mediated by mutations within the deoxycytidine kinase gene. *Cancer Res* 1992, **52**, 2389–2393.
44. Kawasaki H, Carrera CJ, Carson DA. Quantitative immunoassay of human deoxycytidine kinase in malignant cells. *Anal Biochem* 1992, **207**, 193–196.
45. Kawasaki H, Carrera CJ, Piro LD, Saven A, Kipps TJ, Carson DA. Relationship of deoxycytidine kinase and cytoplasmic 5′-nucleotidase to the chemotherapeutic efficacy of 2-chlorodeoxyadenosine. *Blood*, **81**, 597–601.

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