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SHORT COMMUNICATION

Activation of Deoxycytidine Kinase during Inhibition of DNA Synthesis by 2-Chloro-2′-Deoxyadenosine (Cladribine) in Human Lymphocytes

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ABSTRACT. Deoxycytidine kinase (dCK, EC.2.7.1.74), a key enzyme in intracellular metabolism of many antileukemic drugs, was shown to be activated during treatment of lymphocytes by 2-chloro-2′-deoxyadenosine (Cl-dAdo, cladribine), a potent inhibitor of DNA synthesis. While 5-[^{3}H]-thymidine (TdR) incorporation into DNA was decreased by 80–90%, dCK activity was doubled as a consequence of incubating the cells with 1 μM 2-chloro-2′-deoxyadenosine. Thymidine kinase (dTK, EC.2.7.1.21) activity was slightly decreased under the same conditions, similarly to 5-[^{3}H]-thymidine incorporation. dCK activation could not be prevented by cycloheximide, and neither the amount of dCK protein nor its mRNA level was increased after 2-chloro-2′-deoxyadenosine treatment. These results suggest a post-translational activation of dCK protein during inhibition of DNA synthesis. BIOCHEM PHARMACOL **56**;9:1175–1179, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. 2-chloro-2′-deoxyadenosine; cladribine; deoxycytidine kinase; thymidine kinase; human; lymphocyte

dCK (EC 2.7.1.74)§ is a key enzyme supplying the cells with deoxyribonucleotides via the salvage pathway [1]. The enzyme has a lymphoid-specific expression [2–5], but it was also shown in solid tumors [6]. As dCK has a broad substrate specificity [7, 8], the enzyme is responsible for the intracellular phosphorylation of many deoxynucleoside analogues used as anticancer and antiviral drugs, including Cl-dAdo [2, 3], which is highly efficient in the therapy of different leukemias [9, 10]. Cl-dAdo was shown to induce apoptotic cell death [11–13], which is an important and efficient component of chemotherapy [14]. As deoxynucleoside analogues are ineffective without phosphorylation, loss of dCK activity leads to resistance [6]. Efficacy of Cl-dAdo administration was shown to correlate with the *in vitro* phosphorylation capacity of peripheral blood mononuclear cells isolated from leukemic patients [4, 5].

Pretreatment of patients with Cl-dAdo, followed by araC therapy, increased the intracellular level of araCTP [15], offering a possible new way of combination therapy, the elevation of cellular phosphorylation capacity by Cl-dAdo. Preliminary experiments in our laboratory have already shown an increased dCK activity after Cl-dAdo treatment of human tonsillar lymphocytes [16]. Etoposide administration followed by araC therapy was also reported to be an efficient combination, as etoposide increased dCK activity [17].

Regarding the mechanism of dCK activation by Cl-dAdo treatment, one of the possible explanations could be the decrease in dCTP pool sizes, as dCK is subject to feedback inhibition by dCTP [7, 8]. Inhibition of several enzymes, such as ribonucleotide reductase [18], dCMP deaminase [19] and adenosine deaminase [20] by intracellular metabolites of Cl-dAdo, alters the deoxynucleotide pools. However, direct measurement of dCTP pools might be misleading, as compartmentation of dCTP pool was reported in tonsillar lymphocytes [21] and in other cells [22].

In this paper, the activation of dCK will be shown during inhibition of DNA synthesis by Cl-dAdo treatment in human lymphocytes. Furthermore, we present evidence that this enzyme activation cannot be explained by direct allosteric activation of the enzyme or by an elevated expression for the synthesis of dCK, suggesting a new mechanism for the increase in dCK activity.

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§ *Abbreviations:* CdR, deoxycytidine; Cl-dAdo, 2-chloro-2′-deoxyadenosine; dCK, deoxycytidine kinase; dTK, thymidine kinase; and TdR, thymidine.

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MATERIALS AND METHODS

Cells and Treatment

Human tonsillar lymphocytes were isolated from surgically removed tonsils from children (3–6 years) as described earlier [19, 21, 23]. Freshly isolated cells were treated with the drugs in serum-free Eagle's minimal essential medium for 1–3 hr [19, 21]. Cl-dAdo was synthesized by Z. Kazimierczuk [24], and chemicals were products from Sigma, isotopes from Amersham.

Assay of DNA Synthesis

After treatment of cells with 1 μM Cl-dAdo for different times, cells were washed with warm medium, and pulse-labeled with 5-[^{3}H]-TdR (10^7 cells, 1.0 μCi/mL) for 20 min. Radioactivity was measured in the 0.5 N PCA insoluble DNA fraction [19, 21].

dCK-mRNA (Northern Blotting)

Total RNA was extracted from 10^8 tonsillar lymphocytes and purified by ultracentrifugation through $CsCl_2$ gradient [25]. Forty μg RNA/slot was loaded on a denaturing 1.2% agarose-formaldehyde gel, than blotted onto Amersham Hybond nylon filters. The human dCK-specific cDNA probe was excised from pET3D plasmid by BamHl/Ncol digestion [26]. A full-length rat β-actin cDNA probe (a generous gift from L. H. Kedes, University of Southern California) was used as reference. Probes were labeled with α-[^{32}P]-dCTP (110 TBq/mmol) using the random oligonucleotide priming system (Amersham, Multiprime) and purified on Sephadex G-50 columns. Hybridization was performed in 5 × SSC, 5 × Denhardt's, 50% deionized formamide, 2% SDS, 9–10 × 10^6 cpm of probe, 200 μg/mL of chicken DNA and 100 μg/mL of tRNA at 42° for 16 hr, followed by washing for 2 × 10 min, at 20^0 with 2 × SSC, 2% SDS, and for 2 × 5 min, at 65° with 0.2 × SSC, 2% SDS.

Assay of dCK and dTK Enzyme Activity

Crude extracts for dCK and dTK (EC.2.7.1.21) determinations were made according to [27]. Enzyme activities were measured with [^{3}H]dCyd or [^{3}H]dThd as substrates (both 10 μM) in a reaction mixture containing 50 mM TRIS-HCl pH 7.6, 5 mM $MgCl_2$, 5 mM ATP, 2 mM dithiothreitol, 10 mM NaF and extract in a final volume of 50 μL. The mixture was incubated at 37°, and at indicated times aliquots were spotted to DEAE-cellulose filterpapers, which were washed, eluted and counted as described [27].

dCK Protein Level, (Immunostaining)

Electrophoretic separation, Western blotting, and immunostaining by affinity purified primary rabbit antibody against human dCK [28] was performed as described [27], except that Amersham Hybond ECL membranes with the ECL Western blotting analysis detection system (Amersham RPN 2108) were used (secondary antibody in 40,000 × dilution). Purified recombinant dCK was applied as standard.

RESULTS AND DISCUSSION

Increased dCK Activity During Inhibition of DNA Synthesis by Cl-dAdo in Human Lymphocytes

Freshly isolated tonsillar lymphocytes were incubated in the absence or presence of 1 μM Cl-dAdo. At indicated times, the cells were washed and either pulse-labeled with 5-[^{3}H]-TdR for determination of DNA synthesis, or crude cell extracts were prepared and dCK and dTK activities measured [27]. As shown in Fig. 1, 1 μM Cl-dAdo treatment of lymphocytes caused an 80–90% inhibition of DNA synthesis in 2 hr, accompanied by a 100% elevation in dCK activity. The increase in dCK activity in different experiments (cells) varied between 100% and 400%. dTK activity was slightly decreased under the same conditions in parallel to DNA synthesis, as reported previously in primary cultured lymphnode lymphocytes [19]. As Cl-dAdo is activated by dCK [1, 4, 5], an excess (100 μM) of CdR, as a competitive inhibitior of Cl-dAdo phosphorylation, eliminated both the inhibition of DNA synthesis and the activation of dCK (Fig. 1), supporting the intracellular metabolism of Cl-dAdo to exert its effect. Addition of CdR alone had no effect either on dCK or dTK activity, demonstrating that the mithochondrial dTK cannot be responsible for the increased CdR phosphorylation, as this enzyme is using both TdR and CdR as substrates [1, 29]. There was no change in the rate of CdR catabolism under the conditions above, since the amount of [^{3}H]-dCMP added to the crude extracts of treated and untreated cells did not change after 2 hr of incubation (data not shown).

Activation of dCK by Cl-dAdo was not prevented by the presence of 10 μM cycloheximide (Fig. 2), causing *ca.* 50% inhibition of protein synthesis in human tonsillar lymphocytes [23]. Similar results were obtained by 1 mM cycloheximide, which exerted a nearly 100% inhibition of protein synthesis [23] (data not shown).

No Increase in dCK Expression During Cl-dAdo Treatment

In order to determine if there is any change in the dCK-mRNA level, equal amounts of total RNA were applied for gel electrophoresis and hybridization to ^{32}P-dCK-cDNA [26] after 0, 1 and 3 hr of Cl-dAdo treatment of tonsillar lymphocytes (Fig. 3A). No increase, but rather an apparent decrease in the dCK mRNA level was found in control cells (−) as well as in Cl-dAdo-treated cells (+) after 3 hr of incubation. Similar changes were observed in the level of β-actine-mRNA used as reference (Fig. 3A), demonstrating a nonspecific decrease in mRNA level in the primary lymphnode cell cultures.

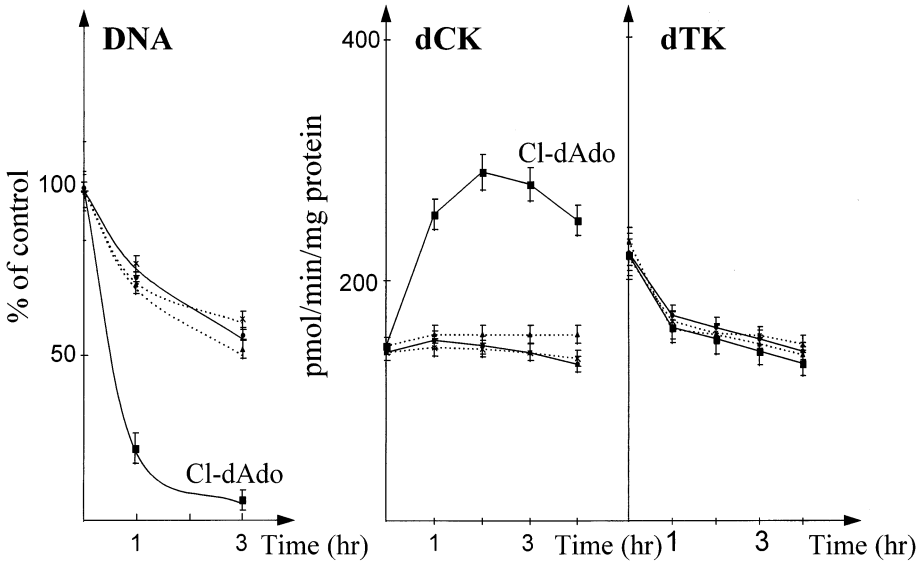

FIG. 1. Alterations of dCK and dTK activities during inhibition of DNA synthesis by Cl-dAdo treatment of human lymphocytes. Human tonsillar lymphocytes were incubated in the absence (—●—) or in the presence of 1 μM Cl-dAdo (—■—), 1 μM Cl-dAdo + 100 μM deoxycytidine (—▲—), and 100 μM deoxycytidine alone (—*—). Then the cells were washed and measured both for the rate of DNA synthesis (DNA) using 5-[^{3}H]-TdR labeling for 20 min, as well as for dCK and dTK enzyme activities, determined from the crude cell extracts as described. 100%: 32000 cpm/10^7 cells.

To compare the level of dCK protein in control (−) and Cl-dAdo-treated (+) cells, crude extracts with equal amounts of protein were separated by gel electrophoresis, blotted and immunostained by human anti-dCK [28], using pure recombinant human dCK as standard (Fig. 3B). Our results show that the amount of dCK protein was constant during Cl-dAdo treatment of cells.

In order to see if any activator or inhibitor could be separated from dCK, the enzyme was partially purified on a DEAE Sephadex column, as described earlier [30]. The degree of dCK activation by Cl-dAdo was found to be the same after purification as in the crude extracts (data not shown), suggesting the change in the enzyme protein itself.

Results presented here show that dCK is activated during the inhibition of DNA synthesis by a short Cl-dAdo treatment of human lymphocytes. This activation cannot be explained either by allosteric activation, by elimination of an inhibitor, or by the new synthesis of the enzyme. It

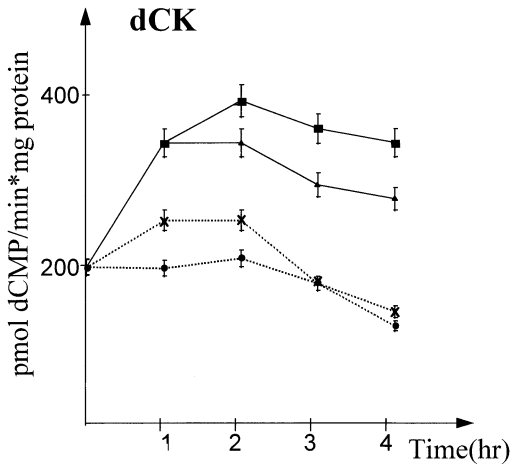

FIG. 2. dCK activation by Cl-dAdo in the presence of cycloheximide. Human lymphocytes were incubated in the absence (—●—) or in the presence of 1 μM Cl-dAdo (—■—), 1 μM Cl-dAdo + 10 μM cycloheximide (—▲—), or 10 μM cycloheximide alone (—*—). dCK enzyme activity was measured as in Fig. 1.

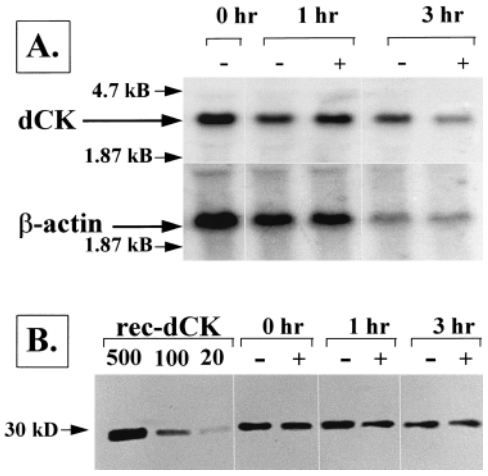

FIG. 3. dCK mRNA and protein levels during Cl-dAdo treatment. Lymphocytes were incubated in the absence (−) or presence (+) of 1 μM Cl-dAdo for 0, 1 and 3 hr. The dCK-mRNA level was determined from total RNA fraction purified by ultracentrifugation, applying 40 μg total RNA in each line. The β-actin-mRNA level was used for comparison and the position of rRNAs are shown by arrows (Fig. 3A). The dCK-protein level was determined by immunostaining with anti-human-dCK, using 40 μg of protein per line from the crude extracts of cells treated as above. Two hundred, 100 and 50 ng of recombinant human dCK was used as reference (Fig. 3B).

seems rather to be an activation of the dCK protein itself, probably occuring through a post-translational modification of the enzyme. We have no evidence that the inhibition of DNA synthesis might be directly correlated to the increase in dCK activity, although they do take place at the same time. Because dCK seems to supply the necessary deoxynucleotides via the salvage pathway mainly for DNA repair [21, 22], its activation might be part of the cellular restoration processes occurring after drug treatment. The elevation of dCK activity by Cl-dAdo might be used as a tool in combination therapy for higher efficacy of other nucleoside analogues. Experiments are in progress to investigate other kinds of toxic agents and/or cellular stresses that could activate dCK beside Cl-dAdo treatment.

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