The potential role of deoxycytidine kinase activation in DNA repair and in apoptosis induction

Doctoral (Ph. D.) thesis

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**Introduction**

DNA replication, repair and recombination require a continuous supply of deoxynucleoside triphosphate precursors. The S-phase-specific *de novo* nucleotide biosynthesis predominates in proliferating cells. In certain cells (e.g. lymphocytes and neurons), however, nucleotides are generated mainly via the salvage reactions which operate both in mitochondria and in the cytosol. The high capacity *de novo* pathway provides deoxyribonucleotides primarily for DNA replication, while salvage systems play a preponderant role in the dNTP supply of DNA repair in resting cells as well as in that of mitochondrial DNA synthesis and repair. Salvage systems save considerable amounts of cellular energy by recycling dietary (extracellular) purine and pyrimidine bases and nucleosides along with those generated during the breakdown of endogenous nucleic acids.

Mammalian cells express four deoxyribonucleoside (salvage) kinases which transfer the terminal phosphate group of either ATP or UTP to the 5’ hydroxyl group of deoxyribonucleosides. Thymidine kinase 1 (TK1) and deoxycytidine kinase (dCK) are located in the cytosol while thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) are in the mitochondria. These enzymes have specific but partly overlapping substrate specificities.

Due to its broad substrate specificity, deoxycytidine kinase is able to phosphorylate not only deoxycytidine but also the purine deoxyribonucleosides as well as several cytotoxic nucleoside analogues that constitute the cornerstone of numerous antileukemic therapeutic protocols. Their phosphorylation by dCK is the rate-limiting step in the bioactivation process that initiates the intracellular accumulation of the corresponding triphosphate metabolites which eventually inhibit DNA synthesis and induce apoptotic cell death. Nucleoside analogues activated by dCK include 2-chloro-2’-deoxyadenosine (CdA), used against hairy cell leukemia and various
lymphomas as well as arabinosyl cytosine (araC), an efficient drug against acute leukemias and 2’, 2’-difluoro-deoxycytidine (dFdC) that has proven to be a potent compound in the treatment of solid tumours. Sufficient levels of deoxycytidine kinase activity in the tumorous tissues are therefore a prerequisite of the clinical efficacy of these nucleoside analogue prodrugs.

Currently, one of the central problems in oncotherapy is the imminent emergence of drug resistance. Clinical resistance of tumours to nucleoside analogues is often caused by loss of deoxycytidine kinase activity or by enhanced levels of the antagonistic 5’ nucleotidase isoenzymes. The chemosensitivity of dCK-deficient and nucleoside analogue resistant tumours might be restored dramatically via transfection of the wild-type dCK cDNA. Recently, a genetically modified, hyperactive dCK variant was engineered that might prove to be a promising tool in gene therapy. These results illustrate that the activity of dCK is one of the main factors influencing the efficacy of chemotherapy. Therefore, increasing the activity of deoxycytidine kinase in cancer tissues by cytostatic drugs and gamma-irradiation might well deserve consideration with regard to the clinical practice, since most tumours have considerable dCK activity that might be further stimulated, while gene therapy with dCK still has a number of unresolved scientific and financial obstacles.
Objectives

My research project was based on a previous observation regarding the specific enhancement of dCK activity in CdA-treated human tonsillar lymphocytes. Since the more active enzyme phosphorylates larger amounts of CdA, the cytotoxicity of the nucleoside analogue might be increased. This phenomenon might bear biochemical importance by providing an insight into the regulation of cellular dCK activity. On the other hand, it might contribute to the development of more efficient chemotherapeutic regimens by selective modulation of the activity of the rate-limiting prodrug kinase. Our aim was to study the molecular mechanism of dCK activation with respect to the following objectives:

1. We wished to clarify whether activation of dCK upon CdA treatment is selective, or it is accompanied by alteration in the activities of other enzymes involved in nucleoside and nucleotide metabolism.

2. We aimed to specify the trigger mechanism of dCK activation by applying physical and chemical cell damage, focusing on the following issues:
   - Is there a difference between various resting and proliferating (normal and transformed) cell populations with regard to the stimulability of their dCK activities?
   - Is there a difference between the effect of natural deoxynucleoside substrates as well as purine- and pyrimidine analogues on the activity of dCK?
   - Do the non-metabolized substrate analogues (nucleoside 5’ thiosulphates) activate the enzyme?
   - Can dCK activity be augmented by targeted DNA-damaging treatments?
Might dCK activation contribute to apoptosis in lymphocytes?

3. We made attempts to uncover the molecular events underlying dCK activation by answering the following questions:

- Is it possible that enzyme activation is brought about by an allosteric mechanism?
- Is it possible that dCK activation is caused by reversible phosphorylation? How is the enzyme activity influenced by disturbances in the kinase/phosphatase equilibrium elicited with protein phosphatase inhibitors and hyperosmotic stress?
- Does dCK undergo a stable conformational change upon activation?
- Does p53 play a role in the activation of dCK upon DNA damage?

**Methods**

**Cell treatments**

Activation of dCK was investigated in primary cell cultures (human tonsillar lymphocytes, normal and leukemic peripheral lymphocytes, thymocytes) as well as in lymphoid and myeloid cell lines (HL60, CCRF-CEM, K562). G- and S-phase enriched cell populations were separated by means of albumin density gradients. Cell suspensions were treated with nucleoside analogues and other genotoxic agents for 1-2 hours. Cells were collected by centrifugation, then washed and extracted by consecutive freeze-thaw cycles. Whole-cell extracts were subsequently applied for enzyme activity determinations and immunoblots. Short-term metabolic labellings of cell cultures were performed by tritiated deoxycytidine and thymidine. Cells were subsequently fractionated by ethanol and radioactivity was counted in the ethanol-soluble pool and in the insoluble (macromolecular, DNA) fraction.
The $\gamma$-irradiation of lymphocytes and semiquantitative determination of DNA fragmentation (Comet assay, single cell alkaline gel electrophoresis) was carried out in collaboration with dr. Géza Sáfrány (National Research Institute of Radiobiology and Radiohygiene).

**Enzyme assays**

Enzyme activities of dCK and thymidine kinases were determined in *in vitro* kinase assays with tritiated deoxycytidine and thymidine substrates, respectively, in the linear kinetic range at saturating substrate concentrations. Deoxycytidylate deaminase and 5’ nucleotidase activities were measured with $^{14}$C-dCMP and $^{14}$C-dIMP substrates, respectively.

**Denaturing and native immunoblot**

dCK protein levels in the cell extracts were compared by Laemmli’s discontinuous SDS-polyacrylamide gel electrophoresis and western blot. Native immunoblots were performed from samples lacking both SDS and denaturing agents, by native electrophoresis and semi-dry electroblot. The dCK-specific rabbit polyclonal antibody raised against the C-terminal peptide was kindly provided by dr. Iannis Talianidis (Herakleion, Greece). Recombinant dCK protein was obtained from dr. Staffan Eriksson (Uppsala, Sweden).

**Determination of deoxynucleoside triphosphate pools and apoptotic markers**

Intracellular deoxynucleoside triphosphate pools were determined from the methanol-soluble supernatant of fractionated lymphocytes according to the method of Sherman and Fyfe, based on primer extension of synthetic oligonucleotide templates. Apoptotic cell death was assessed by measuring caspase 3-like activity of cell extracts and by electrophoretic detection of the fragmentation of genomic DNA (laddering).
Results and Discussion

1. We have shown that out of the deoxyribonucleoside salvage enzymes, only the activity of dCK was increased during short-term (1-2 hrs) treatments of human lymphocytes with CdA. There were no changes in the activities of dCMP deaminase and 5’ nucleotidases, either.

2. Regarding the factors that trigger dCK activation, we made the following statements:

   • By examining dividing and resting cell populations, it turned out that the dCK activity of the G-phase enriched lymphocyte fraction was lower, but it was stimulated to a higher degree than that of S-phase cells, therefore the stimulability of dCK activity and the proliferation rates of normal lymphocytes are indirectly proportional. However, considerable differences were revealed with respect to the degree of dCK potentiation in the leukemic cell lines examined.

   • The extent of dCK activation elicited by deoxyadenosine analogues (CdA) was far larger than that of evoked by pyrimidine derivatives (araC and dFdC), while (ribo)adenosine analogues had only a minimal effect. Of the natural substrates only deoxyadenosine stimulated the enzyme, while deoxycytidine prevented its activation by various agents.

   • The non-phosphorylated thymidine 5’ thiosulphate drastically depleted the thymine nucleotide pool and this event coincided with a significant elevation of dCK activity.

   • Nucleoside analogues have pleiotropic effects but eventually they cause damage to DNA. The causative role of DNA damage in
dCK activation was corroborated by the fact that dCK was profoundly and rapidly activated also by the DNA polymerase inhibitor aphidicolin, the topoisomerase II inhibitor etoposide and, most importantly, by direct DNA damage evoked by gamma-irradiation of cells. There was a tight correlation between the rate of DNA double-strand break repair and the temporal pattern of dCK activation. *We drew the conclusion that stimulation of dCK activity is caused by DNA damage, and elevated levels of dCK activity might be responsible for the more intensive supply of deoxyribonucleotide precursors for reparative DNA synthesis.*

• Activation of dCK was paralleled by increased caspase-3 activities and by selective expansion of the proapoptotic dATP pool. *These results imply that the early activation of dCK might be involved in apoptosis induction in damaged cells by providing more dATP for apoptosome formation.*

3. With regard to the molecular mechanism of enzyme activation, the following data have been obtained:

• Activation of dCK was not followed by elevated protein levels. Moreover, dCK activation could be prevented by treating the cells with BAPTA-AM, a hydrophobic calcium chelator. However, calcium is not a direct cofactor of dCK, since its activity was not decreased in the presence of EGTA. *These findings suggested that dCK activation is brought about by a calcium-dependent post-translational modification of the enzyme.*

• dCK activity could also be elevated by treating tonsillar lymphocytes with calyculin A, a potent inhibitor of protein phosphatase 1 and 2A. Calyculin A and gamma-irradiation synergistically potentiated the activity of dCK. Hyperosmotic stress, known to modify the activity of a range of kinases,
decreased the activity of dCK. *Therefore it seems probable that a reversible protein phosphorylation step is also involved in the process of secondary modification of the dCK protein.*

• A positive correlation has been found between the native immunoreactivity and the enzyme activity of dCK. This is an indirect evidence proving that *dCK undergoes a conformational change during activation*, resulting in an improved accessibility of the C-terminal epitope toward the antibody and leading to *the formation of a catalytically more active steric structure.* The conformational change of dCK was demonstrated by limited tripsinolysis too.
Treating the extracts of CdA-stimulated cells with a recombinant protein phosphatase resulted in a drastic decrease of native dCK immunoreactivity, supporting the assumption that *the conformational change of dCK might have been caused by direct phosphorylation of the enzyme.*
The native recombinant dCK did not react at all with the antibody, suggesting that its steric structure was considerably different from that of its human counterpart.

• p53 plays a seminal role in DNA damage signalling. Pifithrin-?, a recently developed p53 inhibitor, significantly reduced the extent of dCK activation by CdA. Moreover, dCK activity was poorly enhanced in p53 -/- Burkitt’s lymphoma cell lines as compared to those expressing the wild-type p53 protein, *suggesting that functional p53 should be part of the signalling pathway relaying the signal of DNA damage to dCK.*
Publications

Publications related to the theses:


7. **Keszler G**, Spasokoukotskaja T, Sasvári-Székely M, Staub M. Implication of deoxycytidine kinase activation in deoxyadenosine-mediated cytotoxicity. *manuscript, sent for publication*

**Other publications:**


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