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Modifications of deoxycytidine kinase and deaminase activities by docosahexaenoic acid in normal and transformed rat fibroblasts

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Abstract

Deoxycytidine kinase (dCK) and deoxycytidine deaminase (dCDA) are two key enzymes in the activation and inactivation, respectively, of deoxycytidine (dCyd) and several chemotherapeutically important nucleoside analogues. To investigate whether supplementation of docosahexaenoic acid, an *n*-3 fatty acid found mainly in fish oil, can modulate the activity of both enzymes, normal (Rat-2) and transformed (NW-16) rat fibroblasts were cultured in medium supplemented with or without DHA. DHA supplementation increased the phosphorylation efficiency (V_{max}/K_m) of dCK but decreased the deamination efficacy of dCDA in the transformed cells as compared with those in the normal fibroblasts. Enzyme activity of dCK was decreased by DHA in Rat-2 cells and increased in NW-16 cells. In contrast, dCDA activity was elevated in the normal fibroblasts in response to DHA. As a result, the activity ratio of dCK/dCDA (a potential indicator of chemosensitivity) was decreased in the normal fibroblasts but increased in the transformed cells by DHA. We have observed previously that the toxicity of nucleoside drugs (particularly arabinosylcytosine) was increased in tumor cells and decreased in normal cells in response to DHA and proposed a mechanism of changes in drug activation/inactivation. The present data support this hypothesis and suggest that DHA has the potential to selectively target chemotherapeutic drugs toward tumor cells while at the same time reducing host toxicity. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Deoxycytidine kinase; Deoxycytidine deaminase; Fibroblast; Transformed cell; DHA

1. Introduction

Deoxycytidine kinase (dCK) (EC 2.7.1.74) plays an important role in pyrimidine salvage metabolism by converting deoxycytidine (dCyd) to its monophosphate derivative. dCK has gained widespread attention due to its unique role in the rate-limiting phosphorylation of deoxynucleosides and its broad substrate specificity, which enable the enzyme to activate a large variety of chemotherapeutically important nucleoside analogues, such as arabinosylcytosine (araC), cladribine (CdA), and gemcitabine (dFdC) [1]. Clinical resistance to araC has been characterized by decreased *dCK* gene expression [2]. Cell lines selected for resistance to nucleoside analogues showed a lack of *dCK* gene expression [3,4], while transfection of dCK cDNA into those cell lines fully restored sensitivity to these antitumor analogues [5].

dCDA (EC 3.5.4.5) catalyses the irreversible hydrolytic deamination of cytidine and dCyd and their analogues, to the corresponding uridine derivatives and plays a key role in the metabolic inactivation of a number of antitumor and antiviral nucleoside analogues [6]. Increased activity of dCDA results in a significant loss of antiproliferative activity of multiple chemotherapeutic agents [7,8]. Retroviral gene transfer of this detoxifying enzyme has been successful in protecting murine fibroblast and hematopoietic cells from chemotoxicity [9,10]. Therapeutic strategies using dietary manipulation to produce selective toxicity toward tumor cells while protecting normal tissue from drug toxicity could be very useful clinically.

We have demonstrated previously that docosahexaenoic acid (DHA), an *n*-3 fatty acid found mainly in fish oil, has beneficial effects on both the bone marrow compartment

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Abbreviations: dCK, deoxycytidine kinase; dCDA, deoxycytidine deaminase; DHA, docosahexaenoic acid; dCyd, deoxycytidine; araC, arabinosylcytosine; CdA, cladribine; dFdC, gemcitabine; DMEM, Dulbecco's Modified Eagle's Medium.

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and the gastrointestinal tract of tumor-bearing, araC-treated rats [11]. *In vitro* activity of araC was increased in tumor cells but decreased in normal macrophages and fibroblasts following DHA supplementation [12,13]. The mechanism responsible for the selective effect of DHA remains to be elucidated. A recent study demonstrated that the DHA-enhanced cytotoxicity of chemotherapeutic drugs toward tumor cells could be due, in part, to the increased generation of lipoperoxides [14]. However, whether DHA can influence the activation/inactivation process of nucleoside antitumor drugs is not known. Therefore, the present study was designed to investigate whether DHA supplementation can modify drug toxicity through modulation of the activity and protein expression of dCyd kinase and deaminase enzymes in normal and transformed rat fibroblasts.

2. Materials and methods

2.1. Materials

[^{3}H] and [^{14}C]dCyd were purchased from Moravek Biochemicals. Whatman DE-81 filter paper discs came from Fisher Scientific. Dowex 50 (X8) resin was obtained from Supelco. DHA (99.8% purity) was obtained from Nu-Check-Prep, Inc. NW-16 cells expressing the v-*fps* tyrosine kinase were a gift from Ivan Sadowski (University of British Columbia). Polycolonal dCK antibody was a gift from Iannis Talianidis (Institute of Molecular Biology and Biotechnology). dCDA antiserum was donated by Richard Momparlar (University of Montreal). All other chemicals and reagents were of analytical grade and are commercially available.

2.2. Cell culture and DHA treatment

Normal (Rat-2) and v-*fps* transformed (NW-16) rat fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum for 24–48 hr. Then the cells were switched to fresh DMEM supplemented with or without 20 μM DHA and incubated for an additional 48 hr. The DHA concentration chosen was that which produced marked fatty acid compositional changes nearly identical to those produced in tumors and normal tissues from animals fed a DHA-rich diet [11]. In addition, this concentration had little direct cytotoxicity to normal and tumor cells and is physiologically achievable [11,13].

2.3. Enzyme activity assays

The activities of dCK and dCDA were determined in crude cell extracts. Cells were washed three times in PBS and the pellet was resuspended in cold (4°) dCK buffer (0.3 M Tris–HCl, 50 μM β-mercaptoethanol, pH 8.0) or dCDA buffer (0.1 M Tris–HCl, 100 μM EDTA, 100 μM dithiothreitol, pH 8.1). Cell suspensions were then sonicated on ice and centrifuged at 20,000 *g* for 15 min at 4°. The supernatant was used immediately for the enzyme activity assay. Protein concentrations in the supernatant were determined using the Bio-Rad protein assay.

The activity of dCK was measured using a method adapted from Ruiz van Haperen and co-workers [15]. To 25 μL of cell extract in dCK buffer, 25 μL of substrate mixture was added. The substrate mixture was prepared by mixing 2 vol. of Mg^{2+}ATP (50 mM ATP, 25 mM $MgCl_2$, pH 7.4), 2 vol. of [^{3}H]dCyd, and 1 vol. of dCK buffer. The reaction was performed at 37° for 1, 5, 15 and 30 min and terminated by heating at 95° for 3 min. The mixture was then spotted on a DE-81 disk. After washing with 5 mM ammonium formate, the products (nucleotides) were eluted with 0.6 M HCl + 1.5 M NaCl and radioactivity was assessed by liquid scintillation counting.

The activity of dCDA was determined according to the method described by Chan *et al.* [16]. The reaction was conducted by adding 25 μL cell extracts in dCDA buffer to 25 μL of substrate mixture (dCDA buffer + [^{14}C]dCyd) and incubating at 37° for the same time allocations as in the dCK analysis. The reaction was terminated by adding 25 μL of 1 N HCl. The reaction mixture was then applied to a 1 mL Dowex 50 column equilibrated with 0.1 N HCl. The product, deoxyuridine, was eluted by passing 1.5 mL of 0.1 N HCl through the column and radioactivity was assessed by scintillation counting. The substrate concentrations for the activity assays of both enzymes are indicated in "Results." For kinetic analyses, the substrate concentration in the reaction mixture ranged from 5 to 250 μM for the dCK assay and 10 to 1000 μM for the dCDA assay.

2.4. Western blot analysis

Cells were harvested by scraping them from the culture dish, washing them with PBS and resuspending them in PBS containing protease inhibitors (Boehringer Mannheim). Then the cells were sonicated and protein concentrations were determined as described in the activity assay. An equal volume of hot 2× SDS sample buffer (heated to 80°) was added to the cell extracts. Proteins in the cell extracts were separated by SDS-PAGE (12% polyacrylamide gels for the dCK protein assay and 15% gels for the dCDA assay). The separated proteins were then transferred to nitrocellulose membrane using a semi-dry electrotransfer apparatus as previously described [17]. After blocking in a 5% nonfat milk TBS buffer (10 mM Tris, 0.5 M NaCl, 0.4 M KCl) containing 0.05% Tween 20, dCK and dCDA proteins were probed for using their respective polycolonal antibodies diluted in 1% BSA in TBS (1:5000 dilution for dCK antibody and 1:1000 for dCDA antibody). After washing with TBS + Tween 20, membranes were incubated in

horseradish peroxidase-conjugated anti-rabbit IgG diluted in TBS containing 5% milk power and 0.05% Tween 20 (1:50,000 dilution for the dCK assay and 1:30,000 for the dCDA assay). Blots were developed using the enhanced chemiluminescence method. The optical density of the bands was quantified by an imaging densitometer using Northern Eclipse software (Empix Imaging Inc.).

2.5. Data processing and statistics

The reaction velocity of both enzymes was determined by non-linear regression curve-fitting of the time-response for each of the substrate concentrations using Curvefit® computer software (Jandel Scientific). K_m constants and V_{max} values were calculated by non-linear regression analysis using PRISM version 3.0 (Graph Pad). Data were analyzed by one-way ANOVA using an SPSS general linear model program (SPSS Inc.). Analysis of co-variance procedures was applied to control the daily variations within treatment groups. Means were separated by Fisher's protected least significant difference (LSD) test. Differences between means were considered to be significant at $P < 0.05$.

3. Results

Kinetics for dCK and dCDA were determined for Rat-2 and NW-16 fibroblasts treated with or without DHA, using dCyd as substrate and ATP as phosphate donor (Table 1). The K_m values for both enzymes were not different between the two cell lines. The V_{max} values for dCK were higher in the transformed as compared with the normal fibroblasts, while for dCDA they were similar between the two cell types. DHA supplementation increased ($P < 0.02$) the ratio of V_{max}/K_m, an indicator of the efficiency of dCK phosphorylation or dCDA deamination, for dCK but decreased ($P < 0.04$) the ratio for dCDA in NW-16 cells when compared with those in Rat-2 cells.

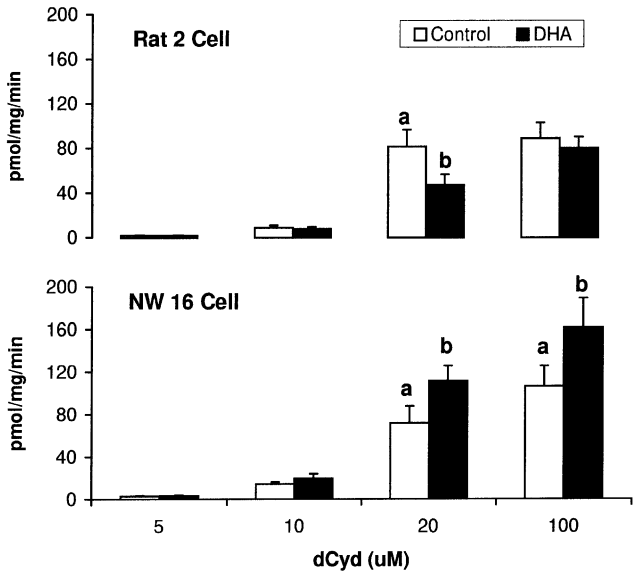

Fig. 1. Modifications of the activities of dCK by DHA. Normal (Rat-2) and v-*fps* transformed (NW-16) rat fibroblasts were cultured in DMEM supplemented with or without 20 μM DHA. Fresh cell extracts were incubated at 37° for 1, 5, 15 and 30 min in reaction mixtures containing graded concentrations of radioisotope-labeled dCyd. The products were separated from the substrates and radioactivity was assessed. The enzyme activities were determined by non-linear regression curve-fitting procedures. Values are means ± SEM of six individual experiments. Means from the same type of cell with different letters in the same substrate concentration are significantly different (one-way ANOVA). The dCyd concentrations (μM) in the reaction mixture are shown on the *x*-axis.

The activity of dCK in Rat-2 and NW-16 cells treated with or without DHA is shown in Fig. 1. DHA supplementation decreased dCK activity in the normal fibroblasts at a substrate concentration of 10 μM or above in the reaction mixture. Such a difference reached statistical significance at 20 μM ($P < 0.035$). In contrast, dCK activity was increased in response to DHA treatment in the transformed cells in all of the four dCyd concentrations tested and was statistically significant at the two higher substrate concentrations ($P < 0.05$).

Table 1
Apparent K_m and V_{max} values of deoxycytidine kinase and deaminase from cells treated with or without DHA

	Rat-2		NW-16	
	Control	DHA	Control	DHA
Deoxycytidine kinase				
K_m (μM)	21.1 ± 2.6	36.8 ± 7.1	33.5 ± 5.7	29.8 ± 6.2
V_{max} (pmol/mg/min)	99.6 ± 6.6 a	98.1 ± 5.4 a	145.6 ± 8.9 b	191.3 ± 18.6 c
V_{max}/K_m	5.1 ± 0.7 ab	3.1 ± 0.5 a	5.2 ± 1.2 ab	7.8 ± 1.6 b
Deoxycytidine deaminase				
K_m (μM)	349.2 ± 12.7	347.0 ± 66.2	462.1 ± 180.1	467.0 ± 90.1
V_{max} (nmol/mg/min)	9.6 ± 3.7	18.2 ± 4.3	10.6 ± 1.8	8.9 ± 1.5
V_{max}/K_m	27.1 ± 6.5 ab	52.3 ± 5.7 a	30.2 ± 12.3 ab	23.7 ± 3.5 b

Cells were cultured in medium supplemented with or without 20 μM DHA. Fresh cell extracts were incubated at 37° for 1, 5, 15 and 30 min in reaction mixtures containing graded concentrations radioisotope-labeled dCyd and ATP. The products were separated from the substrates, and radioactivity was counted. The K_m and V_{max} values were determined by non-linear regression curve-fitting programs. Values are means ± SEM calculated from 4–6 separate experiments. Means with different letters in the same row are significantly different (Fisher's protected LSD).

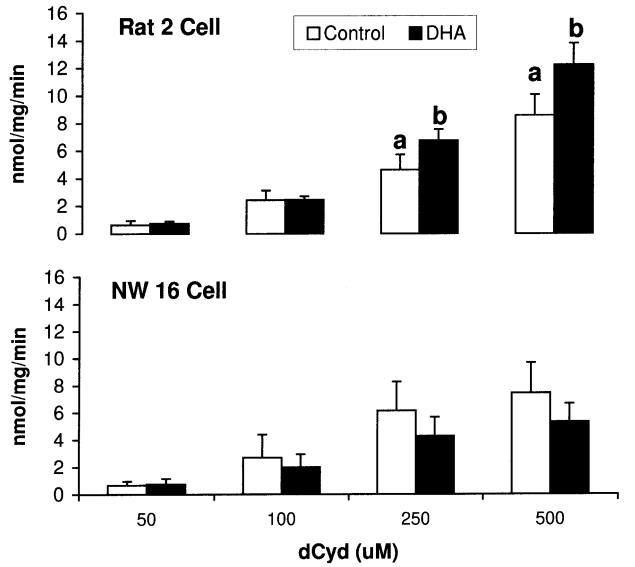

Fig. 2. Modifications of the activities of dCDA by DHA. Normal (Rat-2) and v-*fps* transformed (NW-16) rat fibroblasts were cultured in DMEM supplemented with or without 20 μM DHA. Fresh cell extracts were incubated at 37° for 1, 5, 15 and 30 min in reaction mixtures containing graded concentrations of radioisotope-labeled dCyd. The products were separated from the substrates, and radioactivity was assessed. The enzyme activities were determined by non-linear regression curve-fitting procedures. Values are means ± SEM of five individual experiments. Means from the same type of cell with different letters in the same substrate concentration are significantly different (one-way ANOVA). The dCyd concentrations (μM) in the reaction mixture are shown on the *x*-axis.

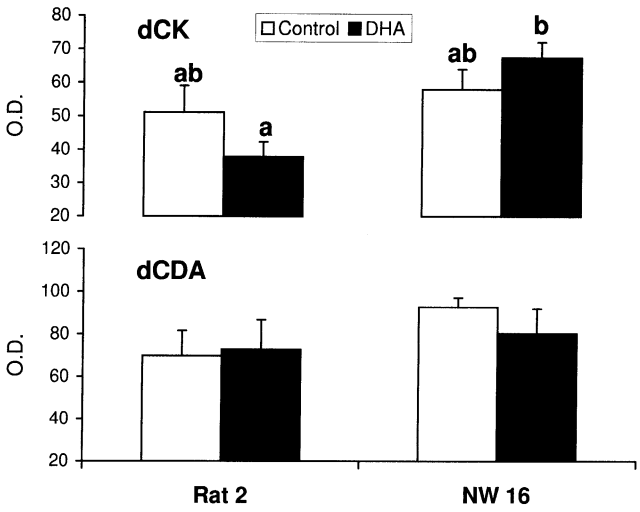

Fig. 3. Protein expression of dCK and dCDA. Proteins in the cell lysates were separated by SDS-PAGE. The separated proteins were then transferred from the gel to a nitrocellulose membrane. After blocking in 5% nonfat milk TBS buffer, the proteins were probed with the primary and secondary antibodies. Blots were then detected using the enhanced chemiluminescence method. The optical densities (OD) of the positive blots were quantified by an imaging densitometer. Values are means ± SEM of seven separate experiments for the dCK protein assay and five experiments for the dCDA assay. Means with different letters for the enzyme of dCK are significantly different (Fisher's protected LSD test).

The activity of dCDA in Rat-2 and NW-16 cells in response to DHA treatment is shown in Fig. 2. The activity of dCDA was increased by DHA supplementation in Rat-2 cells. The differences between control and DHA-treated cells were statistically significant at the two higher concentrations of dCyd ($P < 0.05$). Conversely, dCDA activity appeared lower with DHA in NW-16 cells at the three higher concentrations of substrate, but these differences were not statistically significant.

The protein expression of dCK and dCDA in response to DHA supplementation in the normal and transformed fibroblasts was examined by western blotting (Fig. 3). DHA treatment tended to decrease dCK expression in Rat-2 cells while increasing the amount of this enzyme in NW-16 cells, although such differences were not statistically significant. However, dCK expression was lower in the DHA-treated Rat-2 cells compared with that in NW-16 cells supplemented with DHA ($P < 0.04$). A trend of higher dCDA expression was observed in NW-16 cells compared with normal Rat-2 fibroblasts. However, this difference was not significant.

The ratio of dCK/dCDA activity, a potential indicator of the relative availability of active drug metabolites, in Rat-2 and NW-16 cells in response to DHA supplementation is shown in Fig. 4. The ratio was slightly higher in the transformed cells than in the normal cells at all of the substrate concentrations investigated. DHA supplementation increased the differences in the dCK/dCDA ratio

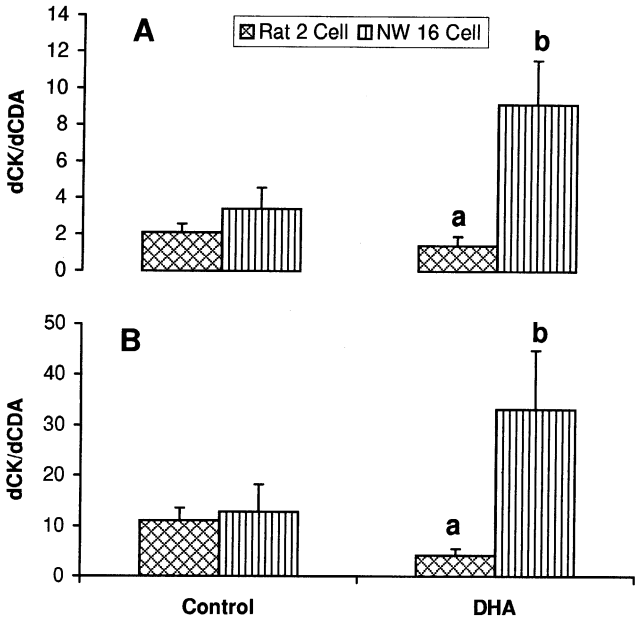

Fig. 4. Modifications of the activity ratios of dCK and dCDA by DHA. The activity ratios of dCK and dCDA from the reactions with substrate concentrations below K_m (10 and 250 μM in the reaction mixture for the dCK and dCDA assays, respectively) are shown in panel A. The activity ratios of dCK and dCDA from the reactions with substrate concentrations around/above K_m (20 and 500 μM in the reaction mixture for the dCK and dCDA assays, respectively) are shown in panel B. Values are means ± SEM of 5 individual ratios. Means with different letters in the DHA treatment groups are significantly different (one-way ANOVA).

between the normal and transformed cells. At dCyd concentrations of 5 and 50 μM in the reaction mixture for dCK and dCDA assays, the ratios were 10.1 ± 4.0 and 2.8 ± 0.4 for the DHA-treated NW-16 and Rat-2 cells, respectively, although differences between them did not reach statistical significance ($P = 0.10$). Increasing the substrate concentration significantly magnified the difference. A 6.7-fold difference was found for the ratio at substrate levels below K_m (10 and 250 μM for dCK and dCDA assays; 9.2 ± 3.8 vs. 1.3 ± 0.4 for the DHA-treated NW-16 vs. DHA-treated Rat-2 cells, respectively; $P < 0.03$) (Fig. 4A). Similarly, a 7.9-fold difference was observed at substrate concentrations around/above K_m (20 and 500 μM for dCK and dCDA assays; 33.2 ± 11.6 vs. 4.1 ± 1.3 for NW-16 vs. Rat-2 cells, respectively; $P < 0.05$) (Fig. 4B).

4. Discussion

The protein of dCK is found primarily in blood cells, macrophages and leukemic cells [18]. Later studies have shown the presence of this enzyme in solid non-lymphoid tissues and tumors [19,20]. dCDA has been found in liver, kidney, spleen and muscle tissue as well as in white blood cells and bone marrow cells [18,21]. dCK plays a critical role at the rate-limiting activation step for several nucleoside antitumor agents, such as araC. Any increase in enzyme activity could enhance the sensitivity and efficacy of a chemotherapeutic regimen [5,22]. Conversely, dCDA is a key enzyme in the metabolic inactivation of a number of antitumor nucleoside analogues. Normal cells can be protected from these cytotoxic drugs by stimulating the activity of dCDA [9]. The present results demonstrated a dual regulatory effect of DHA on the kinetics and activity of both enzymes. DHA supplementation promoted the potency and activity of the enzyme responsible for the activation of dCyd (by analogy we propose that it would function similarly on nucleoside analogues) in transformed cells, while at the same time enhancing the efficacy of the inactivation enzyme in normal cells. As a result, the relative availability of active metabolites (the activity ratio of dCK/dCDA) was about 7-fold higher in the DHA-treated tumor cells as compared with the normal cells.

We have determined the kinetics of dCK and dCDA in the two cell lines using dCyd as the substrate and ATP as the phosphate donor. Most previous studies on dCK kinetics reported an apparent K_m below 10 μM, with the exception of one study reporting an apparent K_m of 300 μM in HL-60 cells [23]. Our values for both cell lines were higher than the commonly reported ones. This discrepancy may be due to the differences in dCK source and experimental conditions. Higher V_{max} values for the dCK in NW-16 cells compared with those in the normal cells could not be explained by increased enzyme expression. Our western blot data showed a significantly higher dCK expression in the transformed cells, but this was seen only in the DHA-supplemented condition. However, V_{max} was different even in unsupplemented cells. Western blotting is incapable of identifying active enzyme, so immunoreactive enzyme levels may not be different but ''active enzyme'' levels may well have differed. Given these observations, DHA may increase enzyme expression while transformation alters the ''active fraction''. DHA supplementation modified the kinetics of both enzymes. The elevated protein expression in the DHA-treated NW-16 cells may contribute to the increased maximum velocity and phosphorylation efficiency of dCK in the transformed cells by DHA. However, the protein level was modified only in dCK and not in dCDA. Thus, a post-transcription mechanism of regulation must be proposed, especially for dCDA.

The activity of dCK can be increased by starvation, administration of dCyd or the steroid (triamcinolone) [24], or treatment with CdA or one of the several inhibitors of DNA synthesis [22]. dCDA activity was elevated by 1,25-dihydroxyvitamin D_3-induced differentiation in a leukemia cell line [25]. The present results demonstrate that lipid supplementation can modulate the activity of these enzymes as well. The dual regulatory effect of DHA on both enzymes suggests a potential for DHA to modulate the level of active nucleoside/nucleotide pools, including those selectively targeting malignant cells. The exact mechanism responsible for such modifications by DHA remains to be elucidated. dCK activity has been reported to be increased by 1.6- to 2-fold during the S phase of the cell cycle [26]. A 1.5-fold increase in concentration of ara-CTP, a phosphorylation product of araC, was observed in a lymphoblastic *T*-cell line during the S phase [27]. In addition, the growth of a DHA-sensitive human melanoma cell line was reported to be arrested by DHA with the cells accumulating within the G_1 or S phase [28]. However, the concentration of DHA used in this study (20 μM) is well below that causing toxicity and there was no sign of direct toxicity by DHA toward either type of cells. Growth curves were similar for both cell lines in the absence or presence of the low concentration of DHA (data not shown). Thus, it seems unlikely that DHA would promote S-phase accumulation in the fibrosarcoma cells rather than the normal cells. DHA could, however, affect post-translational events in enzyme trafficking, subcellular distributing, or other signaling molecules that control drug metabolism or drug action. Experiments are currently underway to identify those alternative mechanisms.

One concern for the current results is that the assays were performed in relatively high substrate concentrations, which may not be physiologically achievable intracellularly. This is especially likely for drugs like araC. In addition, a shift in substrate may also change the kinetics of the enzyme. However, whether the *in vitro* and *in vivo* K_m and V_{max} values are likely to be similar is not known. It is reasonable to speculate that the enzyme would function more optimally in intact cells. Also, certain metabolites may be concentrated in the intracellular compartment at

the sites of enzyme action. We have demonstrated previously that DHA enhanced araC toxicity toward fibrosarcoma and L1210 leukemia cells while protecting normal cells from drug toxicity [12,13]. The present results are very much in line with the previous findings and suggest that differences in activating and inactivating enzymes could explain, at least in part, the differences in toxicity between normal and tumor cells.

In conclusion, DHA supplementation increased the activity of dCK in transformed cells while at the same time enhancing the deamination efficacy of dCDA in normal cells. The modifications by DHA of the enzyme activities, while potentially explaining the differential araC toxicity observed in these cell lines following DHA treatment, cannot be fully explained by the minor changes in enzyme protein expression. The exact mechanism responsible for the observations remains to be elucidated. The present results further support the idea that nutritional interventions, such as lipid supplementation, could be used to help target antiproliferative drugs toward tumor cells and at the same time reduce host toxicity. The scope, in terms of drugs for which this relationship holds, remains to be examined.

Acknowledgments

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