Abstract. Resistance to cytarabine (Ara-C) incapacitates the therapeutic effort during the treatment of acute myeloid leukemia (AML). To elucidate mechanism responsible for the development of resistance to Ara-C, we established the Ara-C resistant AML-2/WT cell sublines, AML-2/IDAC and AML-2/ARC. We then conducted DNA microarray analysis to compare the AML-2/IDAC cells with parental AML-2/WT cells. The results of the microarray analysis revealed a severe defect in the expression of deoxycytidine kinase (dCK), which plays a key role in the transformation of Ara-C to the active form in AML-2/IDAC cells. A similar event was observed in AML-2/ARC cells, but not in Ara-C sensitive AML-2/IDA cells that were resistant to idarubicin. The decreased expression of dCK also resulted in lower activity in both Ara-C resistant variants. However, no significant difference in the intracellular concentration of Ara-C was observed among the cells tested, which indicates that the Ara-C resistant phenotype in our models occurred due to the lower expression and activity of dCK rather than a change in the ability to take up Ara-C. Additionally, in vitro assays using BM cells from AML patients revealed that the expression of dCK and the sensitivity to Ara-C were correlated. Taken together, these findings demonstrate that dCK can regulate the in vitro cellular response to Ara-C in AML cells.

Introduction

Nucleoside analogues have been used for the treatment of a broad range of cancer patients (1). Among these, cytarabine (Ara-C) is a pyrimidine analogue that targets both dividing and resting cells (2). It is also believed that combination treatment comprised of Ara-C and anthracyclines is one of the most effective therapies for patients with acute myeloid leukemia (AML) (3,4). However, even though two thirds of the patients that receive this chemotherapeutic regimen achieve a complete remission (CR), most patients suffer a relapse shortly after entering remission (5,6). Failure of the first chemotherapy and a substantial acquisition of drug resistance also incapacitate the antitumor effect of other chemotherapeutic regimens.

Unlike the cytotoxic mechanism of anthracyclines, the entrance of Ara-C into cells and its subsequent transformation into an active drug are regulated by multiple specific mechanisms (1,2,7). Ara-C enters cells via influx transporters such as the human equilibrative nucleoside transporters (hENTs) and human concentrative nucleoside transporters (hCNTs), after which deoxycytidine kinase (dCK) transforms the Ara-C to Ara-CMP, which is a first step in driving Ara-C to its active form (8). There is growing evidence that abnormal expression and activities in these molecules are involved in both the clinical and in vitro outcomes of Ara-C-dependent chemotherapy. For example, Stam et al and Cai et al reported that high hENT1 levels and defective dCK were correlated with the response to Ara-C in in vivo and in vitro acute lymphoid leukemia (ALL), respectively (9,10). In addition, several studies have revealed that inhibiting enzymes play important roles in the conversion of Ara-C to the active drug (11,12).

In this study, we used two Ara-C resistant AML-2 cell variants, AML-2/IDAC and AML-2/ARC, to elucidate the resistance mechanism against Ara-C in an in vitro AML model. When Ara-C-resistant and -sensitive cells were compared, a severe defect was observed in the expression and activity of dCK in the Ara-C resistant cell lines, which suggests there is a close correlation between the function of dCK and Ara-C resistance in AML. In additional experiments, we found that...
BM cells from an AML patient with a low expression of dCK were less sensitive to Ara-C.

Materials and methods

Cell lines and maintenance. The human leukemia AML-2/WT cell line was obtained from the Ontario Cancer Institute (Toronto, Canada). AML-2/ARC and AML-2/IDA sublines were then established by chronic exposure of the parental AML-2/WT cells to Ara-C and idarubicin, respectively. In addition, an AML-2/IDAC cell variant was generated from AML-2/WT cells as previously described (13). All cells were maintained at 37˚C in a humidified 5% CO2 atmosphere in minimum essential medium alpha (MEM) (Gibco BRL, Grand island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biomeda, Vancouver, Canada) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Gibco BRL).

Patient samples. Bone marrow (BM) aspirates from adults newly diagnosed with AML were collected from St. Mary's Hospital, the Catholic University (Seoul, Korea). Informed consent for the use of all samples was obtained from all patients. No patient received any anticancer therapies prior to the biopsy. A reference BM sample used for RP-PCR analysis was obtained from a healthy donor during BM transplantation. BM samples were separated by Ficoll density gradient centrifugation, after which the mononuclear cells (MNCs) were cryo-preserved in liquid nitrogen. Of the frozen samples, two were thawed and then pre-cultured in RPMI-1640 (Gibco BRL) containing 10% FBS, 2 mM L-glutamine (Sigma, St. Louis, MO) and antibiotics for 24 h. The cells were then used to evaluate the in vitro sensitivity to Ara-C. For RNA extraction, 22 patient samples and normal samples were cryo-preserved using RNAlater (Ambion, Austin, TX) and then stored at -20˚C until use.

Detection of cell cytotoxicity and DNA fragmentation assay. The cytotoxicities of Ara-C against AML-2/WT cells, their resistant variants and BM cells from patients were determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT, USB, Cleveland, OH) assay as previously described (14). Apoptotic cell death was determined based on a DNA fragmentation assay as previously described (15).

Western blot analysis. Following treatment with Ara-C, the cells were lysed and subjected to Western blotting to determine if apoptotic cell death had occurred using a previously described method (16). Anti-caspase-3 and anti-poly-[ADP-ribose] polymerase (PARP) antibodies were obtained from Upstate (Lake Placid, NY, USA). A non-specific band on the X-ray film was used as an internal loading control.

DNA microarray analysis. To compare the gene expression profile between AML-2/WT and AML-2/IDAC cells, we performed DNA microarray analysis using a human whole 35 K oligo chip (GenoCheck, Ansan, Korea), as described previously (17). Briefly, total RNAs from both cell lines were converted into cDNA, after which the generated cDNA samples were hybridized onto the chip. The raw data were then analyzed using GenePix Pro 4.1 (Axon, CA) and Microsoft Excel programs. Final data were expressed as a fold change in the gene expression value in AML/IDAC cells compared to the gene expression of AML/WT cells.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from the cells was extracted using an easy-Blue total RNA Extraction Kit (Intron, Sungnam, Korea), after which the cDNA was synthesized from 1.5 μg of RNA using a Qiagen Omniscript RT kit (Hilden, Germany) according to the manufacturer’s instructions. The primer sequences used for PCR amplification are listed in Table I. All PCR products were separated on a 1.8% agarose gel and then visualized using a UV-transilluminator following ethidium bromide staining. In addition, we conducted an additional experiment using real-time PCR analysis to evaluate dCK expression in BM cells obtained from AML patients. Briefly, each cDNA was mixed with SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA) and then amplified using an ABI PRISM 7900 Sequence Detection System (PE Applied Biosystems). The quantitative data were then analyzed using the Sequence Detection System software (Version 2.0). Next, the expression levels of dCK in each sample were normalized to those of GAPDH according to the comparative cycle time method. The dCK mRNA level from AML patients was expressed as value relative to that of the healthy donor.

dCK activity assay in cell lysate. dCK activity was measured as previously described, with minor modification (18). Briefly, the cells were washed with PBS and then lysed in lysis buffer containing 100 mM NaCl, 50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1% Nonidet P-40, 10% glycerol and protease inhibitors. The cell lysates were then clarified by centrifugation at 14,500 rpm for 15 min, after which their concentrations were estimated using a BCA Protein Assay kit (Pierce, Rockford, IL). The reaction for dCK activity was started by the addition of 125 μg cell lysate into 250 μl of a pre-warmed reaction buffer containing 14.3 pM [5-3H]deoxycytidine (15-25 Ci/mmol/l, Moravek Biochemicals, Brea, CA), 5 mM ATP, 5 mM MgCl2, 50 mM Tris-HCl (pH 7.6), 10 mM NaF, 2 mM DTT and 1.8 mM thymidine. After being allowed to react for 15-30 min at 37˚C, 100 μl of the mixture was pipetted onto a 23 mm DE-81 circle filter paper (Whatman, Maidstone, UK), and then washed three times with ice-cold water to stop the reaction. The level of radioactivity on each filter was then measured by liquid scintillation counting. The radioactivity from a mixture of reaction buffer with lysis buffer was used as a negative control.

Ara-C uptake assay. The cells were seeded at a concentration of 4×103/ml, precultured for 15 min and then resuspended in 500 μl medium. Next, an equal volume of medium containing 1 μCi/ml [3H]Ara-C (15-30 Ci/mmol/l, Moravek Biochemicals) was added, after which the samples were plated into a 96-well plate and incubated for 3 h to allow the cells to take up the treatment. Next, the cells were transferred onto a Filtermat A (Perkin-Elmer, Turku, Finland) using a vacuum device. The filtermat was then washed three times with ice-cold water and dried. The radioactivity of the samples was then measured by liquid scintillation counting.

SONG et al: Ara-C RESISTANCE OF AML CELLS BY LOW EXPRESSION OF dCK
Results

Resistance profile to Ara-C in human leukemia AML-2 cell variants. We established several AML-2/WT cell variants that acquired a drug-resistant phenotype via step-wise exposures to each drug. To characterize the sensitivity to Ara-C in these cells, we performed an MTT assay following treatment with Ara-C. As the Ara-C concentration increased, enhanced cytotoxicities were observed in the parental AML-2/WT cells and their idarubicin-resistant AML-2/IDA variant, but not in AML-2/IDAC and AML-2/ARC cells that were cultured in the presence of Ara-C (Fig. 1A). Furthermore, no detectable change was observed in the cellular DNA of AML-2/IDAC and AML-2/ARC cells that were treated with Ara-C, while DNA laddering representing apoptotic cell death was detected in Ara-C-treated AML-2/WT and -AML-2/IDA cells (Fig. 1B). Additionally, the activation of caspase-3 and degradation of PARP were observed in AML-2/WT and AML-2/IDA cells in response to treatment with Ara-C, but not in the other two variants (Fig. 1C). These results suggest that both AML-2/IDAC and AML-2/ARC cells acquired the potential to protect against Ara-C treatment. This also indicates that a cross resistant phenotype against Ara-C was not present in idarubicin-resistant AML-2/IDA cells, which indicates that they can be used as Ara-C sensitive control cells as well as AML-2/WT parental cells.

Gene expression profiles in drug-resistant leukemia cells. In previous studies, genome-wide approaches have provided a good explanation for the resistant potential of tumor cells to nucleoside analogue drugs (19,20). Therefore, in this study, we evaluated the gene expression profiles using DNA microarray analysis to determine if the difference between both cell lines occurred as a result of the altered expression of genes involved in Ara-C metabolism. Interestingly, a remarkable decrease in dCK expression was observed in AML-2/IDAC cells that were treated with Ara-C when compared to AML-2/WT cells (Fig. 2A). However, there were no significant differences in the expression of other genes associated with Ara-C metabolism, such as activating kinases, deaminase, 5'-nucleotidases and nucleoside transporters, between both cell types. In addition, RT-PCR analyses of the differentially expressed genes confirmed the down-expression of the dCK transcript in AML-2/IDAC cells (Fig. 2B). To determine if the loss of dCK expression is a cell-specific event, the dCK mRNA levels were evaluated by RT-PCR analysis in other drug-resistant variants of AML-2/WT cells. The results revealed that the mRNA of dCK was expressed in AML-2/WT cells and idarubicin-resistant AML-2/IDA cells, while it was not detectable in the other drug-resistant variants of AML-2/WT cells. This indicates that the expression of dCK is a cell-specific event, which suggests that the down-expression of dCK is a critical event in the resistance to Ara-C in AML-2/IDAC cells.

Table I. Primer sequences for PCR amplification.

<table>
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<tr>
<th>Gene</th>
<th>Sequence (5'-3')</th>
<th>Product (bp)</th>
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<tr>
<td>dCK</td>
<td>Fw: GGCCCTGTCACTCTCCTGAG</td>
<td>301</td>
</tr>
<tr>
<td></td>
<td>Re: GCCATCCAGTTATCGGGGA</td>
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</tr>
<tr>
<td>hENT1</td>
<td>Fw: CAACTCTGAGCCCAATG</td>
<td>418</td>
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<tr>
<td></td>
<td>Re: GAGGTAGCGGTGAGAAGGG</td>
<td></td>
</tr>
<tr>
<td>hCNT3</td>
<td>Fw: TCTTTTGAGTTCCATCTCTC</td>
<td>475</td>
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<tr>
<td></td>
<td>Re: TGGGCTCACTCCTTCCTTC</td>
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</tr>
<tr>
<td>CDA</td>
<td>Fw: AAGGCGCTTCAGAAGGGTA</td>
<td>385</td>
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<tr>
<td></td>
<td>Re: GCTAGGAGGGTGCTGACTC</td>
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</tr>
<tr>
<td>dcN</td>
<td>Fw: ACCACACCTGTGGGGTGAG</td>
<td>424</td>
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<tr>
<td></td>
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<td></td>
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<tr>
<td>ß-actin</td>
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<td>309</td>
</tr>
<tr>
<td></td>
<td>Re: CAGGGGTACATGGGTTGGCC</td>
<td></td>
</tr>
<tr>
<td>dCK (real-time PCR)</td>
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</tr>
<tr>
<td></td>
<td>Re: CATCTGGCAACAGGTTGCAGG</td>
<td></td>
</tr>
<tr>
<td>GAPDH (real-time PCR)</td>
<td>Fw: ATGGGGAAAGGTAAGAAGG</td>
<td>108</td>
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<tr>
<td></td>
<td>Re: GGGGTATGGTAGCAACAATA</td>
<td></td>
</tr>
</tbody>
</table>

Fw, forward; Re, reverse; bp, base pair.

Statistical analysis. The results are expressed as the mean ± SD of more than three-independent experiments, each performed in triplicate. Statistical significance of the data was determined using the Student’s t-test. P<0.05 were considered significant.
resistant AML-2/IDA cells, while its expression was defective in Ara-C resistant AML-2/ARC cells (Fig. 2C). These data strongly suggest that dCK expression is closely involved in a chemoresistance to Ara-C that was observed in our in vitro AML cell models.

**dCK activity and Ara-C uptake ability in Ara-C resistant AML-2 cell variants.** To determine if the expression level of dCK affected the ability of cells to convert Ara-C to the active form, we evaluated dCK activities in both Ara-C sensitive and resistant AML-2 cells using [3H]-labeled deoxycytidine. As shown in Fig. 3A, idarubicin-resistant AML-2/IDA cells and parental AML-2/WT cells exhibited increasing activities of dCK as the reaction time was increased to 30 min. However, severe impairment of the dCK activity was observed in both Ara-C resistant AML-2/IDAC and AML-2/ARC cells, which confirms the results of the RT-PCR analysis for dCK.

Figure 1. Sensitivity of AML-2/WT cells and their drug-resistant variants to Ara-C. AML-2/WT (WT) cells and their drug-resistant variants, AML-2/IDA (IDA), AML-2/IDAC (IDAC) and AML-2/ARC (ARC) cells, were cultured in the absence or presence of the indicated concentrations of Ara-C. (A) After treatment for 72 h, the cytotoxicity of Ara-C toward each culture was determined by MTT assay. The results shown are the mean ± SD of three independent experiments. At 24 h after treatment with 5 μg/ml Ara-C, apoptotic cell death in the cells tested was evaluated by gel electrophoresis for DNA fragmentation (B) and Western blot analysis of caspse-3 and PARP (C). A non-specific band is shown as an internal loading control.

Figure 2. Expression levels of dCK in AML-2/WT cells and drug-resistant variants. (A) DNA microarray analysis of representative genes involved in the metabolism of nucleoside analogue in AML-2/IDAC cells. The data are expressed as fold-changes in the transcriptional levels of the genes in AML-2/IDAC cells (IDAC) relative to those of AML-2/WT cells (WT). ND, not determined. (B) Validation of the results from DNA microarray analysis by RT-PCR. β-actin was used as an internal loading control. (C) The transcriptional levels of dCK in both Ara-C sensitive and resistant AML-2 cell lines. RT-PCR analysis was performed using RNA obtained from AML-2/WT, AML-2/IDAC, AML-2/ARC and AML-2/IDA cells. β-actin was used as an internal loading control.
To further explain whether the difference in the response to Ara-C between sensitive and resistant cell lines occurred as a result of differences in the intracellular concentration of Ara-C, we conducted an uptake assay using [3H]-labeled Ara-C. No significant changes were observed among the cells tested (Fig. 3B), indicating that the resistance to Ara-C observed in AML-2/IDAC and AML-2/ARC cells resulted from defects in the activity of dCK, rather than differences in the intracellular accumulation of Ara-C.

To further explain whether the difference in the response to Ara-C between sensitive and resistant cell lines occurred as a result of differences in the intracellular concentration of Ara-C, we conducted an uptake assay using [3H]-labeled Ara-C. No significant changes were observed among the cells tested (Fig. 3B), indicating that the resistance to Ara-C observed in AML-2/IDAC and AML-2/ARC cells resulted from defects in the activity of dCK, rather than differences in the intracellular accumulation of Ara-C.

**Figure 3.** dCK activity and Ara-C uptake in Ara-C sensitive and resistant leukemia cells. dCK activity (A) and Ara-C uptake (B) in both Ara-C sensitive and resistant AML-2 cells were determined as described in Materials and methods. The data are expressed as the mean ± SD from three independent experiments. *P<0.05 vs. AML-2/WT cells.

**Figure 4.** dCK expression and Ara-C sensitivity in BM cells from patients with AML. (A) Total RNA was extracted from BM MNC cells from a healthy donor and 22 patients with AML. The mRNA levels of dCK were quantified by real-time PCR. The values from each specimen were then normalized by comparison with the mRNA values for GAPDH. The dCK levels were then expressed as values relative to that of a normal donor. A dotted line means the dCK level from a healthy donor. (B) Among the 22 specimens, the expression of dCK in Pt. 2 and Pt. 22 samples was confirmed by semi-quantitative RT-PCR. ß-actin was used as an internal loading control. (C) The BM cells from Pt. 2 and 22 were exposed to increasing concentrations of Ara-C for 72 h. The sensitivities to Ara-C were evaluated by MTT assay. The results are expressed as a relative percentage, when compared to the value of the untreated cells. Bar, SD of triplicate values.

**dCK expression and Ara-C sensitivity in BM cells from patients with AML.** Next, the mRNA distribution of dCK was evaluated in BM MNCs from 22 patients newly diagnosed with AML. Quantitative real-time PCR analysis revealed that the dCK levels ranged from 0.563 to 3.679 based on its level in normal counterpart (Fig. 4A). In addition, among the BM specimens obtained from patients, 10 BM specimens exhibited a lower expression of dCK mRNA when compared to that of a healthy donor. To better understand the correlation between
dCK levels and the sensitivity to Ara-C in leukemia cells, we evaluated two patient samples, Pt. 2 and 22, which had a high (2.739) and low (0.563) dCK expression, respectively. The dCK mRNA expressions of these samples were confirmed by semi-quantitative RT-PCR (Fig. 4B). Additionally, the cytotoxicity of Ara-C against both samples was evaluated by MTT assay. We found that BM cells from Pt. 22 were less sensitive to Ara-C than those from Pt. 2, indicating that a low expression of dCK is associated with decreased sensitivity to Ara-C in an in vitro leukemia culture (Fig. 4C).

Discussion

Although a wide range of regimes have been developed for more effective treatment of leukemia, almost all patients with leukemia are treated with nucleoside analogues. Among the nucleoside analogue drugs, Ara-C is regarded as the first choice for patients with acute leukemia (1,21). Although the response rate to initial treatment with Ara-C is high, long-lasting disease-free survival following treatment with this drug seldom occurs due to the development of resistance (22). In the present study, we compared Ara-C sensitive cells to Ara-C resistant variants to elucidate the mechanism that leads to resistance to Ara-C in AML cells. The results revealed decreased expression and activity of dCK in the Ara-C resistant cell group. In addition, an uptake assay conducted to evaluate the intracellular concentration of Ara-C confirmed the involvement of dCK in the drug response observed in our AML cell model.

It is well known that dCK plays a key role in the Ara-C transformation to the active form in vitro, which allows its incorporation into the DNA of the cells (23-25). However, the role of dCK has not been demonstrated in vivo. Previous studies suggested that the dCK level was closely correlated to the response to Ara-C treatment (9,26), which suggests that dCK levels have potential for use as a predictive marker for the susceptibility to Ara-C. In addition, the viral transduction of the dCK gene resulted in remarkable enhancement of Ara-C-induced cytotoxicity in an in vivo study of gliomas (27). However, a study conducted by Veugler et al using mass patient specimens revealed no significant correlation between the expression and activity of dCK and Ara-C response in patients with AML (28). The experimental model utilized in the present study suggested a strong correlation between dCK level and the response to Ara-C. Moreover, comparative analysis of Ara-C metabolism-associated genes using DNA microarray revealed that no other genes were differentially expressed between Ara-C-sensitive and -resistant AML lines. However, these finding can not only explain the Ara-C resistance due to the difference between the cells tested (data not shown), which indicates that the regulation of dCK expression occurs via another mechanism, at least in our AML cell models. Our explanation may be supported by other trials that have defined the reason as mutation or methylation of the DNA encoding the dCK gene (10,33). Taken together, previous studies and the present report indicate that the regulation of dCK expression occurs via a large number of factors.

In conclusion, we evaluated the acquired in vitro resistance and the mechanism by which resistance occurred in AML cells that were exposed to increasing concentrations of Ara-C. The results of this study demonstrated that the defect in dCK activity accompanied by its lower expression was a key mechanism of Ara-C resistance in AML-2 cells. However, further studies are needed to understand the molecular mechanism that leads to the decreased expression of dCK in Ara-C resistant AML cells.

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References


