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Isolation and Preliminary Characterization of 1-β-D-Arabinofuranosylcytosine-Resistant Human Leukemic Cell Line NALM-6

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Abstract

A B cell line of acute human lymphocytic leukemia, NALM-6, was made resistant to 1-β-D-arabinofuranosylcytosine (Ara-C) (NALM-6/Ara-C) by continuously culturing the cells in the presence of increasingly concentrations of Ara-C. In this report, we investigated the involvement of deoxycytidine kinase (dCK) activity in the mechanism by which cells become resistant to Ara-C. The dCK activity was significantly weaker in NALM-6/Ara-C than in NALM-6 cells. NALM-6/Ara-C showed the same restriction pattern of genomic DNA and the same nucleotide sequence of the dCK gene as NALM-6. However, the amount of dCK mRNA was markedly decreased in NALM-6/Ara-C. This is the first report showing in a B cell line of acute human lymphocytic leukemia, NALM-6, that the down regulation of dCK gene expression is affected by a mechanism other than mutation.

Key words — 1-β-D- arabinofuranosylcytosine ; resistance ; deoxycytidine kinase gene ; NALM-6 cells

1-β-D-arabinofuranosylcytosine (Ara-C) is a nucleoside analogue antimetabolite which is an effective agent in the treatment of acute nonlymphocytic leukemia and is also effective in acute lymphoblastic leukemia and lymphoma (1,2). The development of resistance to chemotherapeutic agents is one of the major problems in the treatment of leukemia (3-6). The cytotoxic effect of Ara-C is mediated by its metabolite Ara-CTP, the first step in this phosphorylation being performed by deoxycytidine kinase (dCK). DCK is a pyrimidine salvage pathway enzyme with a subunit molecular weight of 30.5 kDa that uses deoxycytidine and Ara-C among other nucleosides as substrates. The active compound Ara-C is an inhibitor of DNA polymerase. The major cytotoxic mechanisms, however, seem to be mediated by incorporation into DNA thus leading to premature chain termination and slowing of the chain elongation. The extent to which Ara-C is incorporated into DNA is strongly correlated with cell death *in vitro* (1,2,5). A number of studies have reported that, as with most cytotoxic drugs, Ara-C induces cell death via the activation of apoptosis (5,7,8). Concerning the mechanism of resistance to Ara-C, a decrease in the transport of Ara-C, a decrease in dCK activity and an increase in cytidine deaminase activity have been reported, not only in the clinical setting but also *in vitro* (2). A reduction in dCK activity has been suggested to be the mechanism by which human leukemic cells develop resistance to Ara-C (6). The cDNA sequence of human dCK was reported and, in an Ara-C- resistant cell line, two identifiable mutations were detected within the coding region (9). We have previously isolated a hightly Ara-C- resistant human leukemic cell line (NALM-6/Ara-C) with significantly a reduced level of dCK activity (10). In the present study, we examined the mechanism behind the resistance, focusing on the molecure basis for the decrease of dCK activity in NALM-6/Ara-C cells.

MATERIALS AND METHODS

Chemicals

The drugs used in these experiments, Ara-C, etoposide (VP-16), camptothecin (CPT-11) , doxorubicin (DXR), mitomycin C (MMC), 5-

fluorouracil (5-FU) and all the reagents, were of the highest grade available and were supplied by either Sigma (St. Louis, MO, USA), Nakalai Tesque (Kyoto, Japan) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All cell culture reagents were obtained from Invitrogen Corp (Carisbad, CA, USA). Ara-C was dissolved in PBS and stored as a 10 mM stock solution.

Cells and Cell Culture

NALM-6 was obtained from the Cell Resources Center for Biomedical Research, Tohoku University (Sendai, Japan). Cells were routinely kept in RPMI 1640 medium, supplemented with 10% fetal bovine serum and penicillin G (100U/mL)/streptomycin (100 μg/mL) at 37℃ in a humidified 5% CO_2-95% air incubator under standard conditions. All cells were cultured at 4 x 10^5 cells/mL in 100 μL, either in separate wells in 96-well plates for the cytotoxicity assay or in 2 mL in 24-well plates for other assays, and incubated with drugs. The Ara-C-resistant human leukemic cell line NALM-6/Ara-C was obtained by culturing NALM-6 cells with Ara-C gradually increasing the dose. After 6 months, cells which could grow in the presence of 1 μM Ara-C were obtained (10). This cell line has been maintained by alternate feedings with drug-free medium and medium containing 1 μM Ara-C. NALM-6 and NALM-6/Ara-C cells showing logarithmic growth were used in all experiments.

Cytotoxic Analysis

To determine the cytotoxicity of Ara-C in the cell lines, cells were plated in 96-well plates at a concentration of 4 x 10^5 cells/100 μL with various concentrations of Ara-C. After a period, the numbers of cells were determined with the MTT assay (8,11). Briefly, 10 μL of MTT [3-(4,5-dimethylthazole-2-yl)-2,5-dephenyl tetrazolium bromide] solution (5 mg/mL saline) was added to each well. After incubation at 37 ℃ for 4 h, acid-isopropanol (100 μL of 0.04N HCl) was added to each well and mixed thoroughly. The plates were read on a Intermed model NJ-2300 Microplate Reader. Data were expressed as a percentage of the survival of the control cells. The IC_{50} (50% inhibitory drug concentration) was calculated or predicted using our own customized software for each experiment.

Assessment of sub-G1 cells for flow cytometry

Cells (1 x 10^6) were harvested and washed once with cold phosphate-buffered saline (PBS). Then cell pellets were suspended in 500 μL of propidium iodide (PI) solution containing 50 μg/mL of PI, 0.1% (w/v) sodium citrate, and 0.1% (v/v) NP-40. Cell samples were incubated at 4 ℃ in the dark for at least 15 min, and analyzed using a flow cytometer (FACSCalibur, Beckton Dikinson) and Cell Quest software.

Deoxycytidine kinase activity

Deoxycytidine kinase activity was assayed by the method of Spasokoukotskaja et al (12). The incubation mixture (100 μL) contained 50 mM Tris-HCl, pH 7.0, 4 mM ATP, 2.4 mM $MgCl_2$, 1 mg/mL bovine serum albumin, 25 mM dithiothreitol, 130 μM tetrahydrouridine, and 120 nM 5-^{3}H-deoxycytidine (0.5 μCi : 19.3 Ci/mmol ; Amersham, Braunschweig, Germany). The reaction mix was incubated for 20 min at 37 ℃ and heat- inactivated for 2 min at 85℃. Aliquots of 30 μL of the reaction mixture were spotted in triplicate on Whatman DE-81 filter discs. The washing of filters, elution of the radioactive nucleotide formed, and counting procedures were conducted. The enzyme activity is expressed as pmol substrate converted per hour at 37℃ and calculated as per mg

protein present in the cell extract. The amounts of protein were determined according to the method of Bradford (13).

Analysis of the dexoycytidine kinase gene

Total genomic DNA was extracted from NALM-6 and NALM-6/Ara-C cells as described in our previous report (11). Total RNA was purified as described by Chirgwin et al. (14) and poly (A)+RNA was selected using oligo (dT)-cellulose chromatography. Reverse transcription of the mRNA was performed under standard conditions using reverse transcriptase (SuperScript transcriptase, BRL). dCK cDNA was amplified by PCR using the following primers designed on the basis of the known dCK cDNA sequence. The oligonucleotide primers, designed to contain a restriction site for Eco RI or BamHI, were primer P1 (sense) 5'-gggaattcAGCTCTGGGCCGCCACAAGA-3' (nucleotide 133~152) and primer P2 (antisense) 5'-gcggatccCCTGTCACTATA-CACAGATC-3' (nucleotide 542~561), or primer P3 (sense) 5'-gggaattcTGCCT- CTCTGAATGGCAAGC-3' (nucleotide 486~505) and primer P4 (antisense) 5'-gcggatccGGCTGCCTGTAGT-CTTCAGC-3' (nucleotide 947~966). A 429-bp fragment from the 5' half and a 481-bp fragment from the 3' half of the dCK cDNA coding sequence were amplified with P1 and P2, and with P3 and P4, respectively. A 834-bp fragment containing the whole of the coding sequence was amplified with P1 and P4. The amplified products were electrophoresed on 1% agarose gels and stained with ethidium bromide. PCR products from NALM-6/Ara-C cells were the same size as those from NALM-6 cells. The amplified fragments were purified by electrophoresis on 5% polyacrylamide gel. The amplified 834-bp fragment derived from NALM-6 was used as a probe for hybridization. Genomic DNA was digested with Eco RI, Pst I, Bam HI and Hind III, respectively. The digested DNA and poly(A)+RNA were partitioned in agarose gel, transferred to a nitrocellulose sheet or a nylon membrane, and hybridized to random-primer-extended probes (Amersham) as recommended by the suppliers. The amplified 429-bp and 481-bp fragments were subcloned into the vector M13. Single-stranded M13 templates were sequenced by the dideoxy chain termination method (15) with a Sequenase kit (United States Biochemical). At least 10 clones of each amplified DNA were sequenced.

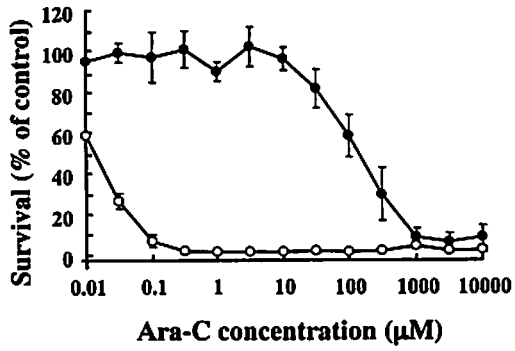

Fig. 1 Inhibitory effect of Ara-C in NALM-6 and NALM-6/Ara-C cells
Dose-response curves of growth inhibition of the NALM-6 (○) and NALM-6/Ara-C (●) cells at the indicated concentrations of Ara-C. Cells were treated with Ara-C for 48 h, and then cell numbers were determined by MTT assay. Cytotoxicity is expressed as a percentage of the untreated cells. Values at each point represent the average of four separate experiments.

RESULTS AND DISCUSSION

Cytotoxic Analysis

The survival of NALM-6 and NALM-6/Ara-C cells exposed to a range of Ara-C concentrations for 48 h, assessed using the MTT assay, is shown in Fig. 1. Ara-C inhibited the growth of NALM-6 and NALM-6/Ara-C cells in a dose-

Table 1 Relative resistance of NALM-6 and NALM-6/Ara-C to antitumor drugs

Drugs	Cytotoxicity (IC_{50})		Relative resistance[a]
	NALM-6	NALM-6/Ara-C	
Ara-C (μM)	0.017 (0.011-0.025)	132.200 (96.874-160.500)	7916.1
VP-16 (μM)	0.361 (0.237-0.551)	0.316 (0.229-0.436)	0.8
CPT-11 (nM)	3.134 (2.412-4.074)	3.341 (2.548-4.382)	1.0
DXR (μM)	0.060 (0.040-0.096)	0.063 (0.042-0.094)	1.0
MMC (μM)	0.154 (0.127-0.188)	0.131 (0.115-0.148)	0.8
5-FU (mM)	0.011 (0.009-0.013)	0.016 (0010-0.025)	1.4

Assessed in quadruplicate by the MTT assay. Each line is the result from one experiment. Values in parentheses are 95% confidence limits. a) Ratio of IC50 (NALM-6/Ara-C)/IC50 (NALM-6). VP-16: etoposide, CPT: camptothecin, DXR: doxorubicin, MMC: mitomycin C, 5-FU: 5-fluorouracil.

dependent manner, but the IC50 for each cell line indicates a 7916.1-fold relative resistance to Ara-C among NALM-6/Ara-C cells compared to NALM-6 cells, but a degree of sensitivity to etoposide (VP-16) , CPT-11 (camptothecin), doxorubicin (DXR), mitomycin C (MMC) and 5-fluorouracil (5-FU) similar to that of the parental NALM-6 cells (Table 1). Since many cytotoxic drugs are known to induce apoptosis of cancer cells (5,7,8), the apoptosis-inducing activity of Ara-C was examined. We treated NALM-6 and NALM/6/Ara-C cells with Ara-C (1 μM) for 24 h, and found an increase in the population of sub-G_1 cells after PI staining, a biochemical hall mark of apoptosis, in NALM-6 but not NALM-6/Ara-C cells (Fig. 2).

Deoxycytidine kinase activity

Resistance to Ara-C can be attributed to many factors but an alteration in the phosphorylation of Ara-C to its active metabolite Ara-CTP by dCK has been widely believed to be the rate-limiting step leading to the incorporation of Ara-CTP into DNA with subsequent induction of DNA strand breaks (5) . This is thought to cause a termination of chain elongation and the switching on of apoptosis via a mechanism not yet fully understood. To investigate whether

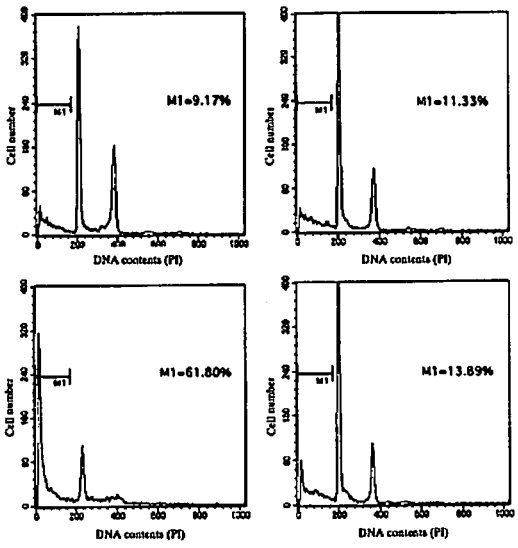

Fig. 2 PI staining and flow cytometric analysis of apoptotic cells
NALM/6 (left panel) or NALM-6/Ara-C cells (right panel) were treated with 0 (upper panel) or 1 μM Ara-C (lower panel) for 24 hr, and then stained with PI, and DNA content was determined by flow cytometry. Apoptotic cell (sub-G_1 cell percentage) populations were marked by M1.

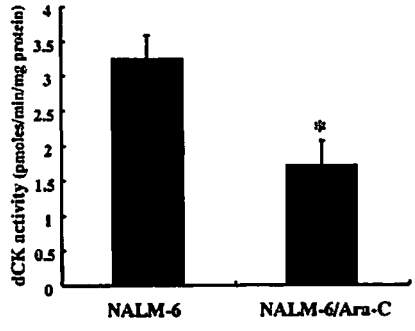

Fig. 3 The activity of deoxycytidine kinase in NALM-6 and NALM-6/Ara-C cells
The results are the mean±S.E. of three separate experiments. * Significant difference from NALM-6 cells at $P<0.05$ with Student's test.

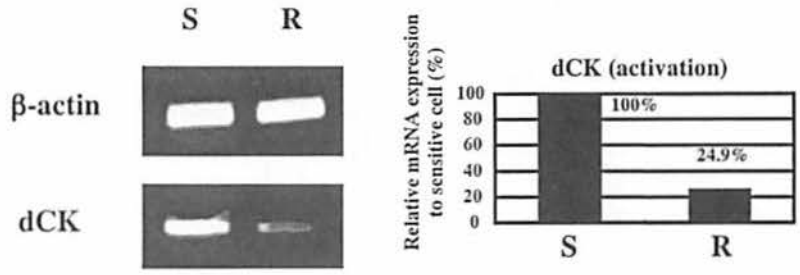

Fig. 4 RT-PCR analysis of the deoxycytidine kinase gene in NALM-6/Ara-C
The dCK mRNA level was determined from the total RNA fraction purified by ultracentrifugation, applying 40 μg of total RNA in each lane. A typical example of ethidium-stained RT-PCR products. The β-actin mRNA level was used for comparison. Lane 1: NALM-6 cells (S) and lane 2: NALM-6/Ara-C cells (R).

the decrease of dCK could be related to the resistance to Ara-C, the activity of dCK was assayed as previously described. Fig. 3 shows the dCK activity of the NALM-6 and NALM-6/Ara-C cells. NALM-6/Ara-C cells showed a reduction in dCK activity (1.70 ± 0.349 nmole/min/mg of protein) in comparison to the NALM-6 cells (3.23 ± 0.345 nmol/min/mg of protein).

Analysis of dCK gene

In this study, NALM-6/Ara-C cells showed a marked decrease in dCK activity. These results suggest that the decrease in dCK activity plays an important role in the *in vitro* model, and recent cloning of the dCK cDNA has allowed investigation of the deficiency of dCK at the molecular level. RT-PCR analysis showed that the amount of dCK mRNA in NALM- 6/Ara-C cells was markedly reduced compared to that in NALM-6 cells (Fig. 4). Analysis of each of the EcoRI, PstI, BamHI and Hind III restriction patterns of genomic DNA revealed no detectable differences between NALM-6 and NALM-6/Ara-C (data not shown). The dCK sequences of NALM-6/Ara-C are identical to those of NALM-6, and are the same as the published dCK cDNA sequence.

The acquired resistance of leukemic cells to Ara-C has already been documented and many mechanisms for this resistance have been postulated (3-6). The deficiency of dCK activity has been considered one of the main mechanisms of resistance, however, the molecular basis for this is still obscure. In 1991, Chottiner et al. cloned the dCK gene cDNA in human T-lymphoblast (MOLT-4) (9), and demonstrated that the dCK mRNA level of Ara-C-resistant cells was much lower than that of the parental cells. The Ara-C-resistant cells have two identifiable mutations in the dCK cDNA coding sequence. One is a point mutation, and the other, a 155-base pair deletion. The expression of each of the two mutant cDNAs in E.coli resulted in a complete loss of catalytic activity (16). No other reports on any mutations of the dCK gene in Ara-C-resistant cells have been published until now.

We examined the molecular change in a highly Ara-C-resistant leukemic cell line (NALM-6/Ara-C) and since the amount of dCK mRNA in NALM-6/Ara-C cells was markedly reduced on Northern blots, it was suggested that the dCK

gene may be expressed at very low levels, or alternatively that the dCK mRNA may be unstable and readily broken. As the restriction patterns obtained with four restriction enzymes on Southern blots were identical between NALM-6 and NALM-6/Ara-C, a wide deletion in the dCK gene was not considered. No mutation was identified in the coding sequences of NALM-6/Ara-C dCK cDNA, suggesting that at least one allele has no mutation in the dCK cDNA coding region. Therefore, it is more likely that the expression of the dCK gene is affected at the level of transcription or mRNA stabilization. If the decrease in transcription causes reduced expression, we also need to evaluate the DNA methylation of the dCK gene (17) as well as the regulatory factors for the expression of the gene. Since the same sequences of the dCK gene were detected in the subcloned PCR products, it seems that the second allele is either not expressed or expressed at extremely low levels in NALM-6/Ara-C cells. A dCK gene mutation which produces unstable mRNA may cause this very weak expression. In order to clarify the cause of the decrease in dCK mRNA, we need to ascertain the sequence of the dCK cDNA 3' untranslated region which may have mRNA stabilized sequences, 5' upstream of the flanking region in which the promoter region lies, and the genomic DNA covering the whole of the cDNA coding region.

In this study we demonstrated that an Ara-C-resistant cell line lacks a mutation of the dCK cDNA coding region, and the results indicated that the decreased activity of dCK does not result only from a mutation in the coding region. In order to clarify the mechanism behind the decrease in activity of dCK in the NALM- 6/Ara-C cell line, further investigation is needed. Although the extend of resistance in the Ara-C-resistant cells that we prepared was very high, dCK activity was reduced by a factor of about 2, suggesting that not only a reduction of dCK activity but also a decrease in intracellular uptake, or an enhancement of cytidine deaminase activity is involved in the mechanism of resistance to Ara-C. In our experiment, the resistant cells showed a decrease intracellular uptake of Ara-C and an enhancement of cytidine deaminase activity (data not shown). This issue is being investigated in detail.

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