Long term cultured HL-60 cells are intrinsically resistant to Ara-C through high CDA activity

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1. ABSTRACT

Cytarabine (araC) is a highly active antimetabolite against hematological malignancy while the agent shows limited activity for some patients despite maintenance or continued therapy with ara-C-containing regiments. In this study, we focused to elucidate the mechanism of intrinsic resistance to araC. The concentration of intracellular ara-CTP and incorporated ara-CTP were monitored in human leukemia cell line-HL-60 for different passages in parental with its variant HL-60R. The expression of mRNA for deoxycytidine kinase (dCK), cytidine deaminas (CDA), human equilibrative nucleoside transporter 1 (hENT1), and cytosolic 50-nucleotidase II (cN-II) were examined by Real-time PCR in HL-60 and HL-60R for different passages. And activities of two metabolizing enzymes for araC, dCK and CDA were further examined. The results showed that the concentration of intracellular ara-CTP was significantly reduced and the ara-U increased in HL-60 cells for 50 passages compared with the 5 passages, and associated with higher CDA activity. All the factors in HL-60R cells did not change by the incubation of ara-C. In conclusion, the long term cultured cells are intrinsically resistant to ara-C through high CDA activity, but not low DCK activity.

2. INTRODUCTION

Cytarabine (araC), a pyrimidine nucleoside analogue, is an antimetabolite commonly used in treatment for acute leukemia (1–3). Ara-C is administered at different doses for effective treatment of different patients. However, there still need many improvements for severe side effects, resistance and so on (4).

Considerable evidence has accumulated the mechanisms of ara-C's bioactivation and action *in vivo*. After being transported into leukemic cells, ara-C is phosphorylated by the deoxycytidine kinase (dCK) (5, 6) to ara-C 50-monophosphate and then to ara-C triphosphate (ara-CTP) (7,8). A small portion of this ara-CTP is incorporated as a monophosphate into DNA strands during the S-phase of the cell cycle (9,10). The drug incorporation into DNA poisons the extending primer against further incorporation of deoxyribonucleotides including dCTP, ultimately inhibiting DNA synthesis (7–10). Meanwhile, the drug is also catalyzed enzymatically to an inactive metabolite,1-beta-D-arabinofuranosyluracil (araU), by CDA (11). Both intracellular ara-CTP and DNA-incorporated ara-C play a critical role for the drug's action (12, 13).

Efficacy of this drug is limited and many patients eventually relapse and die of leukemia despite maintenance or continued therapy with ara-C-containing regiments. However, the precise mechanisms of the intrinsic resistance for ara-C are still unclear. Some studies have disclosed the importance of deoxycytidine kinase (DCK) activity, cytidine deaminase (CDA) activity and the inactive transporters for araC-resistance in leukemia cells (14-18). In this study, we focused on cellular transport and catalytic enzymes in the metabolism by comparisons between leukemia cell line and resistance cell line for long or short cultured time regarding pharmacokinetics and sensitivity. The aim was to determine differences among these groups for further development of novel strategies in leukemia therapy.

3. MATERIALS AND METHODS

3.1. Chemicals and reagents

Ara-C was purchased from Amersham Biosciences (Piscataway, NJ). All other chemicals were purchased from Sigma.

3.2. Preparation of leukemic cells

Human leukemia HL-60 cells were cultured in RPMI1640 media (sigma,st.Louis,MO) with 10% heat-inactivated fetal calf serum (sigma). Its ara-C-resistant variant HL-60/R1, which had been established previously (19,20), parental HL-60 cells were cultured independently, The cells (2 -10^6/ ml, 10 ml) were incubated with various concentrations of ara-C or for different time periods indicated at 37 C.

3.3. Determination of ara-CTP and ara-C/U

HPLC analysis was used to determine intracellular ara-CTP production *in vitro* (21). Briefly, both primary and cultured leukemic cells (1 106 ml_1, 10 ml) were incubated with different concentrations of ara-C for 6 h. Cells were collected by centrifugation (500 g, 5 min, 4 C), followed by extraction of the acid-soluble fraction, the nucleotide pool. The acid-soluble fraction was then applied to the HPLC procedure using a TSK gel DEAE-2 SW column (length, 250 mm; internal diameter, 4.6 mm, TOSOH, Tokyo, Japan) and 0.06 MNa2HPO4 (pH 6.9)—20% acetonitrile buffer. The ara-CTP peak was identified solely by its retention time and quantitated by its peak area at an absorbance of 269 nm. The ara-CTP concentration was expressed as pmol/107 cells.

3.4. Determination of transcript levels of dCK,CDA, cNT-II, and hENT1

To examine mRNA levels of hENT1 , dCK, CDA and cN-II, real-time RT-PCR was performed using the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA). Briefly, total RNA was isolated from each sample (1×10^7 cells) using an RNeasy Mini Kit (Qiagen, Valencia, CA,USA), and subjected to reverse transcription to prepare cDNA using SuperScript-RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitation of the target cDNA and an internal reference gene (GAPDH) was then conducted using TaqMan Gene Expression Assays (Applied Biosystems). The PCRs were performed using TaqMan universal PCR Master Mix according to the manufacturer's instructions. Primers for hENT1, dCK,CDA and cN-II were purchased from Applied Biosystems. The relative standard curve quantitation method was used.

3.5. Enzyme assay

Cells exponentially growing were harvested and disrupted by freezing and thawing to extract cellular proteins. The supernatants after centrifugation had been stored at -80°C until time of measurement. Protein concentration was determined with BCA protein assay kit (Pierce, IL). Activity of DCK was measured by the method described by Durham *et al* (22) after 30 min incubation with (3H)deoxycytidine. That of CDA was determined by the method of Chabner *et al* (23) after 30 min incubation with (3H)araC. The activities were expressed as pmol/min/mg·protein.

3.6. Statistical analysis

Results are expressed as mean±SD. The statistical significance of differences was assessed using t-tests. A value of $p < 0.05$ was considered as significant.

4. RESULTS

### 4.1. Measurement of intracellular and DNA-incorporated ara-CTP in HL-60 cells

To evaluate accuracy, precision, and sensitivity, both intracellular ara-CTP production and DNA-incorporated ara-CTP were quantitated in ara-C-treated HL-60 cells. Figure1 demonstrated that the concentration of intracellular ara-CTP were higher than that DNA-incorporated ara-CTP, and both ara-CTP increased at concentration- and time-dependently, reaching a plateau at concentrations of 5 to 10 mM or at incubation periods of 4 to 6 h. According to this precise and accurate quantitation of intracellular and DNA-incorporated ara-C in biologic materials, incubation with 8uM ara-C for 4 h was used in the following investigatin.

4.2. Intracellular ara-CTP, ara-C and ara-U concentrations in HL-60 and HL-60R

To further investigate whether the cells acquired resistance for long term cultured, we confirm the concentration of ara-CTP, ara-C and ara-U in HL-60 and HL-60R for different passages. After incubated with ara-C, the concentrations of the intracellular ara-C were low in both cell lines, and there were no significant change for long term cultured. The production of ara-CTP was significant low in the HL-60R compared with the HL-60, indicating a critical role of ara-CTP in ara-C induced cytotoxicity. However, the concentration of the ara-CTP in HL-60 reduced significantly to the similar level of HL-60R for 50 passages, but the ara-U conversely increased to a high level, indicating the ara-C was catalyzed enzymatically to an inactive metabolite in the long term cultured cells (Figure 2-A). The concentration of ara-CTP and ara-U in HL-60R were reduced slowly

for long term cultured (Figure 2-B).
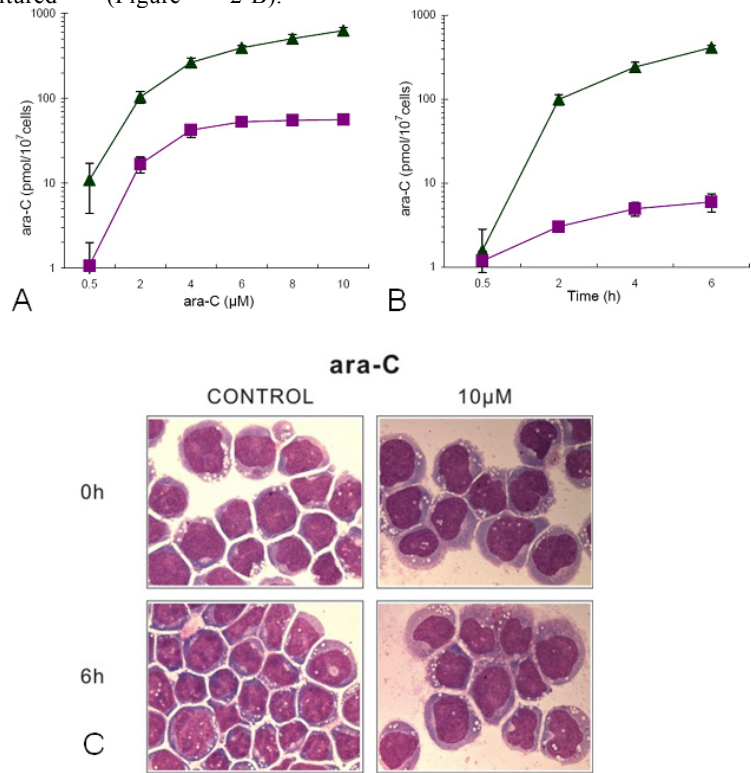
Figure 1. The intracellular ara-CTP and incorporated ara-CTP in human leukemia cell line-HL-60. A: the HL-60 cells were incubated for 6h with various concentrations (0.5,2, 4,6,8,10uM) of ara-C in media. B: the HL-60 cells were incubated with 8uM ara-C for timecourse (0.5, 2, 4, 6h). C. Morphology of HL-60 incubated with 10uM ara-C for timecourse 6h.
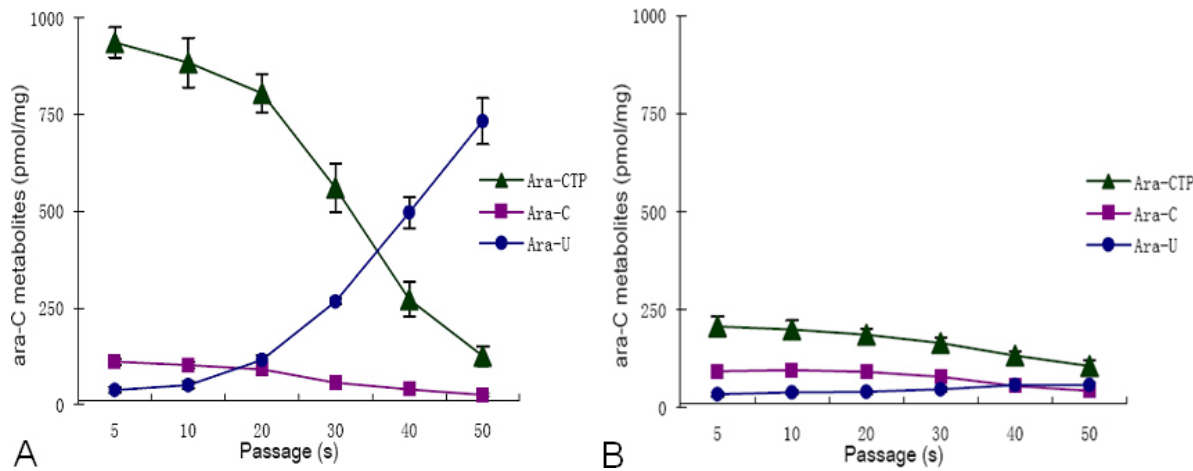
Figure 2. The intracellular ara-CTP, ara-C,ara-U in human leukemia cell line-HL-60 and HL-60R. A: the HL-60 cells of different passages were incubated for 4h with 8uM of ara-C in media. B: the HL-60R cells of different passages were incubated with 8uM ara-C for for 4h.

4.3. The expression of ara-C-related factors in HL-60 and HL-60R cells

The transcript levels of dCK, CDA, hENT1, cNII were evaluated in HL-60 and HL-60R respectively. Figure 3 showed the dCK and hENT1 expressed higher in HL-60 cells than the HL-60R cells, the cNT-II was opposite, and there were no little increase or decrease of these three factors between different passages cells. However, the

mRNA expression level of CDA in HL-60 was increased linearly and reached the higher level than the HL-60R,
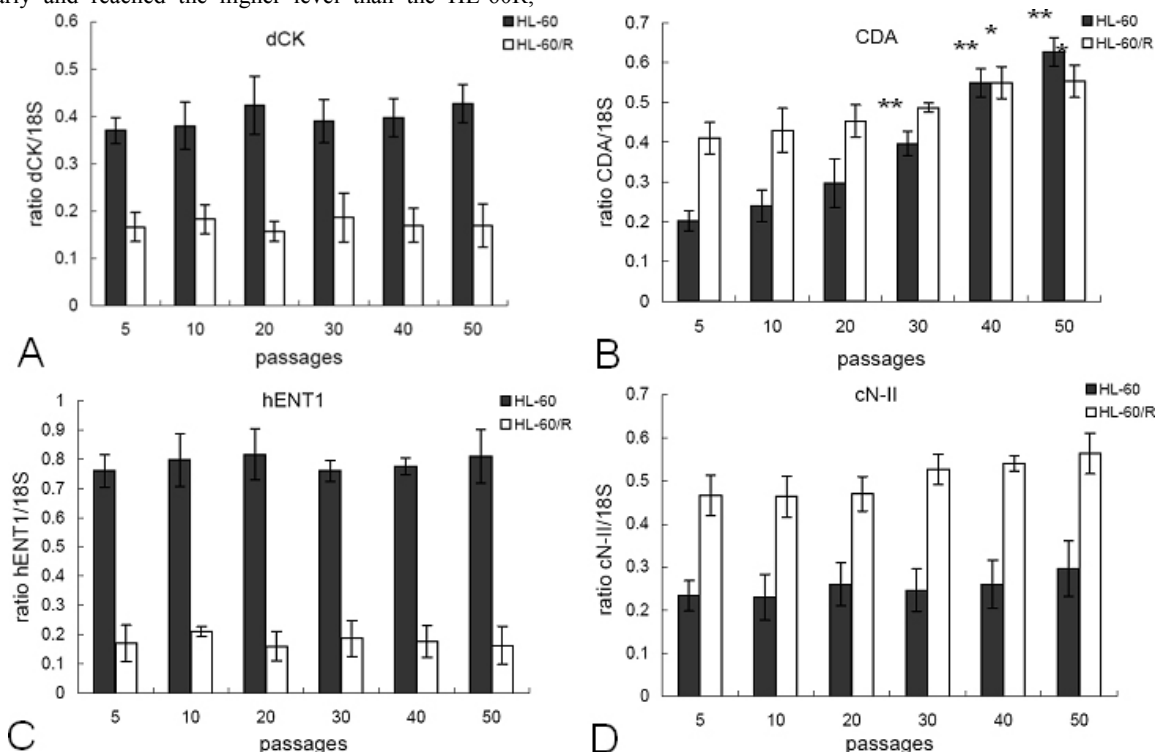
Figure 3. The mRNA expression of dCK (A), CDA (B), hENT1 (C) and cNT-II (D) in HL-60 and HL-60R cells for different passages (5,10,20,30,40,50 passages). Columns represent mean data (+SD).The black represents the HL-60 cells, the white represents the HL-60R cells. Significant differences: *p<0.05, **p<0.01 compared to 5 passages.
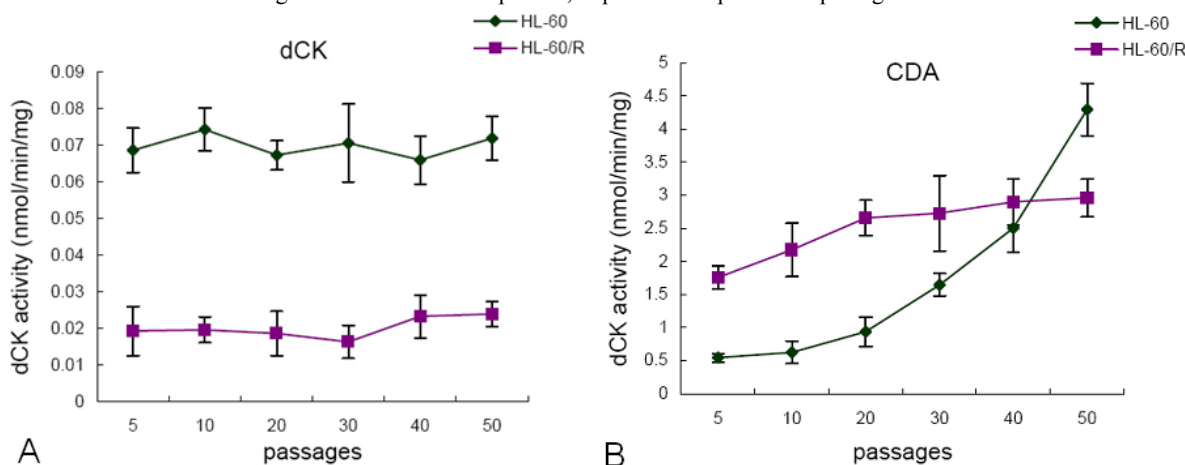
Figure 4. Activities of ara-C metabolizing enzymes in HL60 and HL60R cells. Cellular protein was extracted and measured for enzyme activity of dCK (A) or CDA (B). The green represents the HL60 cells and the pink represents the HL60R cells. Bar is the SD of three determinations.

indicating that the increased expression of CDA may play an vital role for the high level of ara-U for the long term cultured.

4.4. Determination of enzyme activity involved in metabolism of Ara-C

Although the expression of the dCK and CDA were examined, the enzyme activity were needed further determined for their direct role during the ara-C metabolism, for 5 passages, the HL-60R cells demonstrated significantly lower DCK and higher CDA activities than the parental HL-60 cells (0.02 vs. 0.07 nmol/min/mg·protein in dCK activity and 2 vs. 0.05

nmol/min/mg·protein in CDA activity) (Figs. 4A and 4B). Moreover, during the cell culture passages, there's a significant increase of CDA activity in HL-60 cell, only a little change in HL-60R cell. But, there's no significant alternation detected in dCK activity in both cell lines for different passages.

5. DISCUSSION

The efficient formation of intracellular ara-CTP is indispensable for obtaining cytotoxic response after ara-C treatment (24–25). In this study, the pharmacokinetics of ara-CTP in HL-60 and HL-60R cells for different passages were compared. To investigate differences, an extracellular concentration of 8 uM ara-C for 4h was selected at which formation of ara-CTP was saturated in all cells and avoided side effects.

The initial steps of araC metabolism, phosphorylation by dCK and deamination by CDA, these steps are reported to greatly influence accumulation of the active metabolite (26-29). Also, we measured other determinants such as transporter hENT1 and cNT-II, which is one of the seven 50-nucleotidases that catalyze the dephosphorylation of ribo- and deoxyri-bonucleoside monophosphates, thereby counteracting the action of nucleoside kinases (30),. in order to assess their exact values. The results revealed that dCK activity was low in HL60R cells than the HL-60 cells, means that ara-C entering cells can be phosphorylated to their active metabolites more effectively in HL-60 cells. Conversely, CDA activity was higher in HL60R cells which agreed with the mRNA expression. The more important is that the CDA activity significantly increased for the long term cultured cells. From these results, treatment strategies against resistance should include modulation of the high CDA activity. Combination of CDA-inhibitors may overcome the resisitance. The development of novel agents with low substrate specificity to CDA is also promising. In addition, high CDA activity in resistant can be a target of chemotherapy.

6. CONCLUSION

In conclusion, this study indicated that the long term cultured HL-60 cells were associated with high CDA activity but not by low dCK activity. These results may be helpful for development of new deoxycytidine analogues or treatment strategies against resistance or relapse of human acute leukemia patients.

6. ACKNOWLEDGEMENTS

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