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Intracellular cytarabine triphosphate production correlates to deoxycytidine kinase/cytosolic 5′-nucleotidase II expression ratio in primary acute myeloid leukemia cells

Takahiro Yamauchi *, Eiju Negoro, Shinji Kishi, Kazutaka Takagi, Akira Yoshida, Yoshimasa Urasaki, Hiromichi Iwasaki, Takanori Ueda

Department of Hematology and Oncology, Faculty of Medical Sciences, University of Fukui, 23 Shimoaizuki, Matsuoka, Eiheiji, Fukui 910-1193, Japan

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ABSTRACT

Cytarabine (ara-C) is the key agent for treating acute myeloid leukemia (AML). After being transported into leukemic cells by human equilibrative nucleoside transporter 1 (hENT1), ara-C is phosphorylated to ara-C triphosphate (ara-CTP), an active metabolite, and then incorporated into DNA, thereby inhibiting DNA synthesis. Deoxycytidine kinase (dCK) and cytosolic 5′-nucleotidase II (cN-II) are associated with the production of ara-CTP. Because ara-C's cytotoxicity depends on ara-CTP production, parameters that are most related to ara-CTP formation would predict ara-C sensitivity and the clinical outcome of ara-C therapy. The present study focused on finding any correlation between the capacity to produce ara-CTP and ara-C-metabolizing factors. In vitro ara-CTP production, mRNA levels of hENT1, dCK, and cN-II, and ara-C sensitivity were evaluated in 34 blast samples from 33 leukemic patients including 26 with AML. A large degree of heterogeneity was seen in the capacity to produce ara-CTP and in mRNA levels of hENT1, dCK, and cN-II. Despite the lack of any association between each of the transcript levels and ara-CTP production, the ratio of dCK/cN-II transcript levels correlated significantly with the amount of ara-CTP among AML samples. The HL-60 cultured leukemia cell line and its three ara-C-resistant variants (HL-60/R1, HL-60/R2, HL-60/R3), which were 8-, 10-, and 500-fold more resistant than HL-60, respectively, were evaluated similarly. The dCK/cN-II ratio was again proportional to ara-CTP production and to ara-C sensitivity. The dCK/cN-II ratio may thus predict the capacity for ara-CTP production and ultimately, ara-C sensitivity in AML.

1. Introduction

Cytarabine, or 1-β-D-arabinofuranosylcytosine (ara-C), is the key agent for treating acute myeloid leukemia (AML). The combination of regular-dose ara-C given for 7 days with daunorubicin given for 3 days has been a standard induction therapy for AML. This regimen achieves complete remission rates of ≥70% in adult AML patients [1,2]. However, these remissions are not durable, and long-term survivors account for only 30–40%. To improve clinical outcomes, individualization of treatment is needed according to prognostic factors including chemosensitivity in each patient. In this regard, the sensitivity of leukemic cells to ara-C may be crucial for predicting therapeutic efficacy and establishing tailor-made chemotherapy.

As the mechanism of action, ara-C is transported into leukemic cells by membrane transporters including the human equilibrative nucleoside transporter 1 (hENT1) [3]. Inside the cell, ara-C is phosphorylated by the rate-limiting enzyme deoxycytidine kinase (dCK) to ara-C monophosphate, and then to ara-C diphosphate by deoxycytidine monophosphate kinase and eventually to ara-C triphosphate (ara-CTP), an active metabolite of ara-C, by nucleoside diphosphate kinase [4]. Ara-C catabolism results from rapid deamination by cytidine deaminase to the non-toxic metabolite ara-U [4], while cytosolic 5′-nucleotidase II (cN-II) dephosphorylates ara-CMP [5], thereby preventing production of the active form. Ara-CTP is then incorporated into DNA strands during the S phase of the cell cycle, thereby inhibiting DNA synthesis [6–8]. As drug incorporation into DNA is the product of the ara-CTP concentration and time, ara-CTP levels represent an index of ara-C cytotoxicity. Furthermore, the clinical value of intracellular ara-CTP level has been established with the identification of a

* Corresponding author. Tel.: +81 776 61 3111; fax: +81 776 61 8109.
E-mail address: tyamauch@u-fukui.ac.jp (T. Yamauchi).

Abbreviations: ara-C, cytarabine or 1-β-D-arabinofuranosylcytosine; ara-CTP, cytarabine triphosphate or 1-β-D-arabinofuranosylcytosine triphosphate; AML, acute myeloid leukemia; hENT1, human equilibrative nucleoside transporter 1; IC_{50}, 50% growth-inhibitory concentration; dCK, deoxycytidine kinase; cN-II, cytosolic 5′-nucleotidase II; XTT, sodium 3′-(1-[(phenylamino)-carbonyl-3,4-tetrazolium])-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate.

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correlation between the intracellular pharmacokinetics of ara-CTP and response to ara-C therapy [9–13].

If the capacity of patients' leukemic cells to produce ara-CTP is determined prior to therapy, sensitivity to ara-C-based chemotherapy and ultimately the clinical outcome could be predicted. As the production of ara-CTP depends on the cellular function of ara-C metabolism in each patient's leukemia, previous studies sought to identify any relationship between clinical outcomes of ara-C-based chemotherapy and ara-C-related factors including hENT1, dCK, and cN-II [14–18]. However, results were inconsistent, since the studies dealt with patients having different genetic backgrounds [14–18]. More importantly, no exact correlation was revealed between the capacity to produce ara-CTP and ara-C-metabolizing factors in leukemic cells. The factors that are functionally most crucial to the production of ara-CTP thus remain undetermined.

We hypothesized that factors that can correlate with ara-CTP production may offer a predictor for ara-C sensitivity and its therapeutic efficacy. The present study focused on finding any correlation between the capacity to produce ara-CTP and ara-C-metabolizing factors in patients' leukemic cells. For this purpose, we quantitated the in vitro production of intracellular ara-CTP, mRNA expression levels of hENT1, dCK, and cN-II, and ara-C sensitivity in blasts from leukemic patients including those with AML. In addition, we evaluated four cultured leukemia cell lines with different sensitivities to ara-C in parallel experiments.

2. Materials and methods

2.1. Patient samples

The present study was approved by the ethics committee of the University of Fukui Hospital, and informed consent was obtained from each patient. A total of 34 leukemic cell samples from 33 leukemic patients were evaluated (Table 1). One patient was evaluated at diagnosis and again at relapse after the first remission following induction chemotherapy. The evaluated leukemia included 26 cases of AML, 4 cases of acute lymphoblastic leukemia, 2 cases of plasma cell leukemia, and 1 case of leukemic manifestation of peripheral T cell lymphoma (Table 1). The diagnosis of leukemia was made by standard cytological and histochemical examination of bone marrow smears according to the French–American–British criteria [19].

Table 1
Patient characteristics.

No. of patients evaluated	33
Sex (*n*)	
Male	17
Female	16
Age (years)	
Median	69
Range	18–86
Diagnosis	Number
ML-LM	1
ALL (L2)	3
CLL	1
PCL	2
AML (M0)	2
AML (M1)	7
AML (M2)	12
AML (M4)	2
AML (M5)	2
AML (M7)	1

No. or *n*, number; years, age; ML-LM, leukemic manifestation of malignant lymphoma; ALL, acute lymphoblastic leukemia; PCL, plasma cell leukemia; AML, acute myeloid leukemia; M0-7, subclass of FAB classification.

Prior to chemotherapy, peripheral blood was drawn into heparinized tubes, layered over Ficoll-Hipaque (Beckman-Couler Japan, Tokyo, Japan), and centrifuged ($500 \times g$, 30 min, room temperature) to isolate leukemic cells [9]. The cells were washed twice with phosphate-buffered saline and then centrifuged ($500 \times g$, 5 min, 4 °C) to pellet the cells. Aliquots were resuspended in RPMI1640 media supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a 5% CO_2-humidified atmosphere for further experiments.

2.2. Preparation of cultured leukemic cells

This investigation used human leukemia HL-60 cells and its three ara-C-resistant variants, HL-60/R1, HL-60/R2, and HL-60/R3, which had been established previously [20,21]. In brief, to develop ara-C-resistant variants, parental HL-60 cells were cultured independently in three flasks in media containing ara-C. The initial concentration of ara-C was half the 50% growth-inhibitory concentration (IC_{50}) for HL-60 cells. Cultures were observed daily and allowed to grow. Drug concentrations on subsequent passages were gradually increased, and one cell line resistant to ara-C was cloned from each flask using the limiting dilution method. The three independent ara-C-resistant HL-60 variants were named HL-60/R1, HL-60/R2, and HL-60/R3. Reduced dCK activity was demonstrated in R1 and R2 cells, while increased cN-II activity was also present in R2 cells [20,21]. R3 cells almost lacked dCK activity [20,21]. These cell lines were maintained in RPMI1640 media supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a 5% CO_2-humidified atmosphere.

2.3. Determination of intracellular ara-CTP

HPLC analysis was used to determine intracellular ara-CTP production in vitro [9]. Briefly, both primary and cultured leukemic cells (1×10^6 ml^{-1}, 10 ml) were incubated with different concentrations of ara-C for 6 h. Cells were collected by centrifugation ($500 \times g$, 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 M Na_2HPO_4 (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/10^7 cells.

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

To evaluate mRNA levels of hENT1 (accession: NM_001078177), dCK (accession: NM_000788), and cN-II (accession: NM_012229), 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, and cN-II were purchased from Applied Biosystems. The relative standard curve quantitation method was used. The values for cultured human leukemia K562 cells were set as controls, and values of given samples were determined as the ratio compared with the value of K562 cells. We used K562, because its transcript levels were closer to those in patients' leukemic cells than those in HL-60 were.