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A sensitive new method for clinically monitoring cytarabine concentrations at the DNA level in leukemic cells

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Abstract

Cytarabine (ara-C), a major antileukemic agent, is phosphorylated in the cell to cytarabine triphosphate (ara-CTP), which is then partly incorporated into DNA. The drug incorporation into DNA poisons the extending primer against further incorporation of deoxyribonucleotides including dCTP, ultimately inhibiting DNA synthesis. While intracellular ara-CTP concentration has been found to predict clinical outcome, cytotoxicity in vitro is determined primarily by the extent of drug incorporation into DNA. However, clinically appropriate quantitation methods for ara-C at the DNA level have not been available. We developed a sensitive new method for monitoring ara-C incorporated into DNA in vivo. After DNA from leukemic cells was fractionated using the Schmidt-Thannhauser-Schneider method, it was degraded to constituent nucleosides to release ara-C, which was isolated from the nucleosides using HPLC and then measured by radioimmunoassay. Recovery for DNA fractionation, ara-C release by degradation, and ara-C isolation were 92.0 ± 6.4%, 90.7 ± 9.4%, and 98.5 ± 1.4%, respectively. The method was found to determine ara-C incorporation into DNA of ara-C-treated HL 60 cells in vitro with minimal interassay variation. The values determined were compatible with those determined by scintillation counting in parallel experiments using tritiated ara-C. Our method could be used to monitor DNA-incorporated ara-C concentrations during ara-C therapy, together with plasma ara-C and intracellular ara-CTP concentrations. ara-C incorporation into DNA appeared to be associated with intracellular retention of ara-CTP or persistence of plasma ara-C. Thus, the present method is sensitive, accurate, precise, and may permit therapeutic drug monitoring at the DNA level for better individualization of antileukemic regimens.

Keywords: Cytarabine; DNA incorporation; Cytarabine triphosphate; Acute leukemia; TDM; Individualization

1. Introduction

ara-C, a pyrimidine nucleoside analogue, is among the most effective anticancer agents for treatment of acute leukemia [1–3]. The drug offers flexibility of use including doses at 3–10 mg/m^2 (low dose), 70–100 mg/m^2 (regular dose), and 1–3 g/m^2 (high dose) more than a 1000-fold range [3–6]. Mechanisms of bioactivation and action are uniform regardless of dose. After being transported into leukemic cells, ara-C is phosphorylated to ara-C 5′-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]. Therefore, both intracellular ara-CTP and DNA-incorporated ara-C are metabolites critical to the drug's action [7–10].

In in vitro studies, both intracellular ara-CTP and DNA-incorporated ara-C correlated with the cytotoxic effect of ara-C [7–12]. However, the correlation between ara-C incorporated into DNA and cell death was more significant than that between ara-CTP and cytotoxicity [9,10,13]. Incorporation into DNA is also critical to the cytotoxic activity of fludarabine and gemcitabine [14], suggesting general importance of interactions between DNA and nucleoside analogues. Clinically observed therapeutic efficacy correlated closely with intracellular ara-CTP during high-dose ara-C treatment [15–20]. At a regular dose, we developed a sensitive method and confirmed that the metabolite ara-CTP was crucial to therapeutic outcome

Abbreviations: ara-C, cytarabine or 1-β-D-arabinofuranosylcytosine; ara-CTP, cytarabine triphosphate or 1-β-D-arabinofuranosylcytosine triphosphate; TDM, therapeutic drug monitoring; STS, Schmidt-Thannhauser-Schneider

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[21–23]. As for DNA-incorporated ara-C, one report described an assay method and its use to monitor ara-C at the DNA level in the clinic [24]. Yet, no studies have been conducted subsequently, reflecting need for more suitable in vivo monitoring methods. Therefore, DNA-incorporated ara-C measurement has not yet been evaluated for clinical efficacy despite its critical role for in vitro cytotoxicity.

To determine the clinical significance of DNA-incorporated ara-C, a better method is needed for monitoring ara-C concentrations at the DNA level accurately and sensitively in clinical samples. Ideally the method would allow simultaneous measurement of intracellular ara-CTP and plasma ara-C.

In the present study we developed a sensitive new method for monitoring ara-C incorporated into DNA in vivo. The procedure included fractionation of DNA from leukemic cells (step 1), degradation of DNA to nucleosides to release ara-C (step 2), isolation of ara-C using HPLC (step 3), and measurement of ara-C by radioimmunoassay (step 4). The present study focused first on validation of recovery for each step of the assay procedure. Next, accuracy and precision of the method were confirmed using cultured leukemic cells in vitro. Last, clinical utility was demonstrated by applying the method to monitoring ara-C concentrations within the DNA of peripheral leukemic blasts obtained from patients receiving ara-C.

2. Materials and methods

2.1. Chemicals and reagents

ara-C, bacterial alkaline phosphatase (EC 3.1.3.1), snake venom phosphodiesterase (EC 3.1.4.1), and DNase I (EC 3.1.21.1) were purchased from Sigma. [5-^{3}H] ara-C (30 Ci/mmol), [methyl-^{3}H]thymidine (20 Ci/mmol), [5-^{3}H]uridine (20 Ci/mmol), and [thymidine-methyl-^{3}H]-DNA (0.2 μCi/μg) were purchased from Daiichi Pure Chemicals. Tetrahydrouridine, a cytidine deaminase inhibitor, was obtained from Calbiochem-Novabiochem. Charcoal was purchased from J.T. Baker Chemical. Anti-ara-C serum was kindly supplied by Daiichi Pure Chemicals. This antiserum did not cross-react with phosphorylated forms of ara-C, uracil arabinoside, cytidine, or deoxycytidine [25]. All other chemicals were of analytical grade.

2.2. Preparation of leukemic cells

Human leukemia HL 60 cells were cultured in RPMI1640 media with 10% heat-inactivated fetal calf serum. The cells (2×10^6 ml^{-1}, 10 ml) were incubated with various concentrations of ara-C for the time periods indicated at 37 °C. Cells were then washed twice with PBS and centrifuged (500 × *g*, 5 min, 4 °C) to collect the cell pellet.

2.3. DNA fractionation from cells

To fractionate DNA from the cell pellet, the classical STS method was used with slight modification [26–28]. The cell pellet described above was added to 30 μl of 15% perchloric acid in a microcentrifuge tube. The sample was then vortexed, cooled in an ice bath for 15 min, and centrifuged (15,000 × *g*, 30 s, 4 °C). The supernatant (acid-soluble fraction) was discarded, while the precipitate was obtained as the acid-insoluble fraction. To solubilize the RNA, the acid-insoluble fraction was resuspended in 100 μl of 0.4N KOH and kept at room temperature for 4 h. The sample was then mixed with 100 μl of 5% perchloric acid and 20 μl of 4N HCl, followed by centrifugation (15,000 × *g*, 30 s, 4 °C). After removal of the supernatant (RNA), the precipitate was mixed with 100 μl of 5% perchloric acid, and then heated at 92 °C for 20 min to solubilize the DNA. After centrifugation (15,000 × *g*, 30 s, 4 °C), the supernatant was isolated as DNA while the precipitate (protein) was discarded.

To assess DNA fractionation, a pellet of HL 60 cells (1×10^7 cells) mixed with 10 μl of [thymidine-methyl-^{3}H]-DNA underwent extraction of the acid-insoluble fraction and the subsequent fractionation of DNA. Radioactivity of the fractionated DNA was compared with that of the DNA mixed with the cell pellet. To further assess the fractionation procedure, cells (2×10^6 ml^{-1}, 10 ml) were incubated with 20 μl of [methyl^3-H]thymidine or ^{3}H-uridine for 5 h at 37 °C. The cell pellet was subsequently fractionated into DNA, RNA, and protein, and radioactivity of each fraction was measured.

2.4. ara-C release by degradation of DNA to nucleosides

To release ara-C from DNA, the DNA was degraded to the corresponding nucleosides. The DNA fraction described above was once added to 70 μl of 1N KOH, vortexed, and centrifuged (15,000 × *g*, 30 s, 4 °C) to obtain the supernatant as the neutralized DNA. The neutralized DNA solution was then mixed with 300 μl of 1 M Tris, 5 μl of 10 mM $CaCl_2$, 5 μl of 10 mM $MgCl_2$, 250 U of DNase I, 60 μg of phosphodiesterase, 10 U of alkaline phosphatase, and 10 μl of 1 mg/ml tetrahyrdouridine. This mixture (pH 8) was incubated for 12 h at 42 °C to digest the DNA to nucleosides.

To confirm degradation, HL 60 cells (2×10^6 ml^{-1}, 10 ml) were incubated with an aliquot of tritiated ara-C for 5 h at 37 °C, followed by fractionation of DNA. To separate ara-C, the DNA sample was spotted on a TLC sheet (POLYGRAM CEL 300 UV_{254}, 20 cm × 20 cm × 0.1 mm, Machery-Nagel) before or after the degradation procedure, and developed by a 2-propanol:ethyl acetate:-water (2:2:1) solvent for 3 h. Radioactivity of ara-C released from DNA after degradation was compared with that of ara-C-containing DNA before degradation.

2.5. ara-C isolation using HPLC

To isolate ara-C from other nucleosides, HPLC was employed. Nucleosides obtained as described above were injected onto a cation-exchange column (Partisil 10 SCX, 250 mm length × 4.6 mm inside diameter; Whatman) and eluted with 0.01 M ammonium formate (pH 3.0) at a constant flow rate of 1.5 ml/min, at ambient temperature. ara-C was identified by its retention time (15.5 min) at 254 nm. The ara-C fraction was collected from 13 to 18 min for a 5-min interval, and was then freeze-dried overnight.

2.6. ara-C measurement using radioimmunoassay

To measure ara-C concentration, radioimmunoassay was carried out as previously described [21,25]. This was a competitive reaction between tritiated ara-C and unlabeled ara-C for binding the antiserum. Briefly, the ara-C fraction obtained above (unlabeled, unknown for its concentration) was dissolved in 400 μl of 0.01 M phosphate buffer containing 0.5% bovine serum albumin and 0.9% NaCl (pH 7.4), 100 μl of anti-ara-C serum, and 100 μl of diluted tritiated ara-C (0.05 μCi). The mixture was incubated for 3 h at 37 °C to allow competition between the labeled and the unlabeled for binding the antibody. To separate the bound and free ara-C, the sample was added to 1 ml of dextran-coated charcoal (100 mg of dextran and 1000 mg of charcoal per 100 ml of the phosphate buffer above), cooled for 30 min at 4 °C, and centrifuged (15,000 × *g*, 30 s, 4 °C). The supernatant (the antibody-bound ara-C) was isolated, and its radioactivity was counted on the following day. The ara-C concentration of the sample was extrapolated in the standard curve that was drawn simultaneously using 10 independent known concentrations of ara-C. The lower limit of quantitation for the radioimmunoassay was 0.1 pmol.

2.7. Drug monitoring at the DNA level in the clinic

Blood samples were obtained with informed consent from four leukemic patients receiving ara-C. The patients were diagnosed with leukemia or leukemic transformation of myelodysplastic syndrome by standard criteria with bone marrow examination. All were refractory or relapsed. In two patients (Nos. 3 and 4), plasma ara-C and intracellular ara-CTP concentrations were monitored simultaneously. Each blood sample was placed in heparinized tubes containing tetrahydrouridine at a final concentration of 0.1 mM, layered over Ficoll-Hipaque, and centrifuged (500 × *g*, 30 min, room temperature) to separate plasma and leukemic cells [29,30]. Plasma ara-C concentration was determined using the radioimmunoassay described above [21,25]. Blasts were washed twice with PBS and then centrifuged (500 × *g*, 5 min, 4 °C) to pellet the cells. The pellet (2×10^7 cells) was divided into acid-soluble (supernatant) and acid-insoluble (precipitate) fractions by adding 30 μl of 15% perchloric acid. The acid-insoluble fraction was used to measure ara-C incorporated into DNA as described above. The acid-soluble fraction was used to measure intracellular ara-CTP as previously reported [21]. Briefly, the acid-soluble fraction was neutralized with KOH and applied to an ion-exchange column (TSK gel DEAE-2 SW, 250 mm length × 4.6 mm inside diameter; TOSOH). Elution was performed using 0.05 M Na_2HPO_4 (pH 6.9)–20% acetonitrile at a constant flow rate of 0.7 ml/min, at ambient temperature. ara-CTP was monitored spectrophotometrically at 269 nm, fractionated, and freeze-dried overnight. After ara-CTP obtained was dephosphorylated enzymatically, the resulting ara-C was measured using the radioimmunoassay described above (Fig. 1).

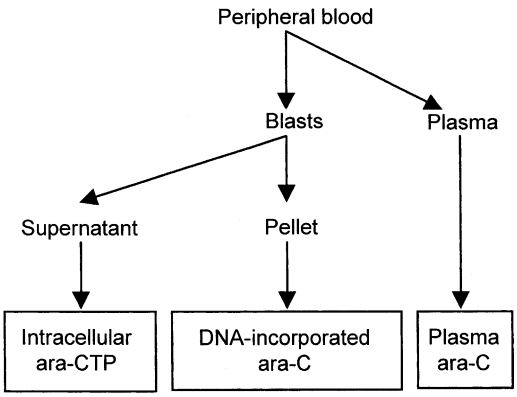

Fig. 1. Scheme for clinical monitoring of ara-C and metabolites. Plasma ara-C, intracellular ara-CTP, and DNA-incorporated ara-C can be measured simultaneously in one blood sample.

2.8. Measurement of DNA-incorporated ara-C and intracellular ara-CTP using tritiated ara-C

To verify the present method, cultured HL 60 cells or primarily obtained leukemic cells (2×10^6 ml^{-1}, 10 ml) were incubated with tritiated ara-C in parallel experiments. Cells were then washed twice with PBS, and centrifuged (500 × *g*, 5 min, 4 °C). The pellet was divided into acid-soluble and acid-insoluble fractions by adding 30 μl of 15% perchloric acid. DNA was fractionated from the acid-insoluble fraction using the STS method, while ara-CTP was isolated from the acid-soluble fraction using HPLC as described above. Radioactivity of each metabolite was counted on the following day.

2.9. Statistical analyses

All statistical analyses were performed with Microsoft Excel 2003 (Microsoft Corporation). All graphs, linear regression lines, and curves were generated using GraphPad Prism (version 4.0; GraphPad Software).

Table 1
Recovery at each step

Step in procedure		Radioactivity (DPM)		Recovery (%)
(1) DNA fractionation (1)				
		Before	After	
		37194	33291	
		37732	32879	92.0 ± 6.4
		38260	38000	
(2) DNA fractionation (2)				
	RNA	DNA	Protein	
Thymidine	330 (2%)	14802 (90%)	1308 (8%)	DNA (%)
	944 (6%)	13900 (92%)	306 (2%)	91.6 ± 1.6
	902 (7%)	12820 (92%)	53 (1%)	
Uridine	27563 (91%)	1102 (3%)	1848 (6%)	RNA (%)
	29766 (90%)	3055 (9%)	349 (1%)	90.0 ± 0.1
	28885 (90%)	2871 (8%)	514 (2%)	
(3) ara-C release				
		DNA	ara-C	
		2300	1864	
		2000	1995	90.7 ± 9.4
		2496	2281	
(4) ara-C isolation				
		Before	After	
		38200	38213	
		38380	37380	98.5 ± 1.4
		39324	38571	

(1) A pellet of HL 60 cells mixed with tritiated DNA underwent the DNA fractionation procedure. Radioactivity of the fractionated DNA was compared with that of the DNA mixed with the cell pellet. (2) Cells were incubated with tritiated thymidine or uridine, followed by fractionation into DNA, RNA, and protein. Radioactivity of each fraction was measured. (3) Cells were incubated with tritiated ara-C, and then DNA was fractionated from the cells and subsequently degraded to nucleosides. Radioactivity of ara-C released from DNA after degradation was compared with that of ara-C-containing DNA before degradation. (4) Tritiated ara-C and standard deoxyribonucleosides were co-eluted in HPLC. ara-C was fractionated, lyophilized overnight, and recovered in 1 ml of water. Radioactivity of ara-C recovered was compared with that of ara-C applied to HPLC. Values for recovery are the means ± S.D. of paired samples analyzed in triplicate.

3. Results

3.1. Recovery in the assay steps

Recovery was evaluated for the steps of DNA fractionation, ara-C release, and ara-C isolation. To assess recovery at the step of DNA fractionation, the pellet of HL 60 cells was mixed with tritiated DNA, followed by the DNA fractionation procedure described in Section 2. Radioactivity of DNA fractionated from the mixture was equal to that present in the tritiated DNA added to the cell pellet (Table 1). To further confirm the completeness of the fractionation procedure, the cells were incubated with tritiated thymidine or uridine, followed by fractionation into DNA, RNA, and protein as described in Section 2. Radioactivity in each fraction demonstrated clear separation and excellent recovery of DNA (Table 1).

To assess recovery at the step of ara-C release, cells were incubated with tritiated ara-C to allow its incorporation into DNA. The DNA was then fractionated from the cells and ultimately degraded to nucleosides as described in Section 2. TLC revealed that radioactivity of ara-C released from DNA after degradation was compatible with that of the ara-C-bearing DNA before degradation (Table 1).

To assess recovery at the step of ara-C isolation, a mixture of tritiated ara-C and standard deoxyribonucleosides was eluted by HPLC. ara-C (retention time: 15.5 min) was clearly separated from the other nucleosides (retention times: uracil arabinoside, 2.3 min, thymidine, 2.7 min, 2′-deoxyguanosine, 6.5 min, 2′-deoxycytidine, 23.3 min, 2′-deoxyadenosine, 34.8 min), and subsequently fractionated, lyophilized overnight, and recovered. Radioactivity of the ara-C recovered was equal to that of the ara-C added to the mixture (Table 1). Thus, recovery was excellent at each step in the present assay procedure, strongly supporting its practicability.

3.2. Measurement of DNA-incorporated ara-C in biologic samples

To evaluate accuracy, precision, and sensitivity of measurements in biologic samples, DNA-incorporated ara-C was quantitated in ara-C-treated HL 60 cells using our assay. Fig. 2 demonstrates that DNA-incorporated ara-C increased both concentration- and time-dependently, reaching a plateau at concentrations of 5 to 10 μM or at incubation periods of 4 to 6 h. As for inter-assay variability, the coefficient of variation was approximately 30% at each concentration (Table 2), suggesting

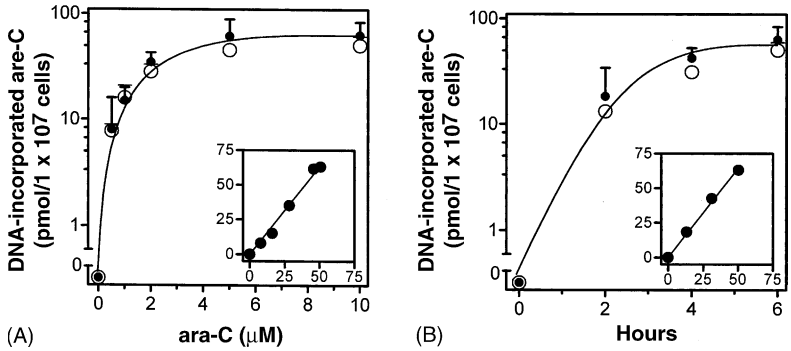

Fig. 2. Measurement of DNA-incorporated ara-C in biologic samples in vitro. HL 60 cells were incubated with various concentrations (0.5, 1, 2, 5, and 10 μM) of ara-C for 6 h (A), or with 10 μM ara-C for the indicated time periods (2, 4, and 6 h) (B). ara-C incorporated into DNA was measured using the present assay method (●). Alternatively, the cells were incubated with tritiated ara-C in the same experimental setting, and ara-C incorporated into DNA was then determined by scintillation counting (○). Inset: DNA-incorporated ara-C concentrations were compared between these two measurements for each experiment. Ordinate: DNA-incorporated ara-C measured by the present method (pmol/1 × 10^7 cells). Abscissa: DNA-incorporated ara-C measured by scintillation counting (pmol/1 × 10^7 cells). $r^2 = 0.99$, slope = 1.16, $P < 0.0001$ for (A) inset, $r^2 = 0.99$, slope = 1.12, $P < 0.0001$ for (B) inset.

Table 2
Interassay variation for quantitating ara-C incorporated into DNA

ara-C (μM)	DNA-incorporated ara-C		
	Mean	S.D.	CV (%)
1	15	4.0	27.2
2	35	7.1	20.2
5	61	22.4	36.5
10	63	18.8	29.9

HL 60 cells were incubated for 6 h with the indicated concentrations of ara-C on three separate days. Concentrations of ara-C incorporated into DNA (pmol/1 × 10^7 cells) were measured using the present method. CV: coefficient of variation.

appropriate precision despite the multistep nature of the method and the use of biologic materials. The lower limit of quantitation, defined as the lowest concentration that gave a coefficient of variation less than 50%, was 1 pmol/1 × 10^7 cells. This limit permitted sufficient sensitivity to detect DNA-incorporated ara-C yielded by incubation with ara-C at 0.5 μM (Fig. 2A), a concentration near the plasma concentration obtained during continuous infusion of conventional-dose ara-C [23]. To evaluate accuracy of the method, ara-C concentrations at the DNA level measured by the present method were compared with those obtained using tritiated ara-C in the same experimental setting (Fig. 2A and B). ara-C concentrations obtained by these two methods were quite similar to each other, with statistically significant correlation values (Fig. 2A and B, inset). Thus, the present assay method was precise, accurate, and sensitive for quantitation of DNA-incorporated ara-C in biologic materials, strongly supportive of clinical applicability.

Table 3
ara-C incorporated into DNA of leukemic cells from patients receiving ara-C

Patients	Age/gender	Diagnosis	Treatment	WBC (blasts %) (μ/l)	Time[a]	DNA-incorporated ara-C (pmol/1 × 10^7 cells)
1	50/F	AML (M2) [First relapse]	1 g/m^2, 1 h-iv inf	40000 (98)	1 h (day 1)	60
					2 h (day 1)	40
			3 g/m^2, 2 h-iv inf	17300 (98)	3 h (day 1)	110
2	72/F	MDS-LT [Primary refractory]	1 g/m^2, 1 h-iv inf	64800 (87)	5 h (day 1)	15
				46600 (89)	5 h (day 2)	35
3	73/M	AML (M5b) [First relapse]	1 g/m^2, 1 h-iv inf	60000 (97)	0 h (day 1)	3.7
					1 h (day 1)	3
					3 h (day 1)	1.9
					5 h (day 1)	ND
					8 h (day 1)	ND
4	59/M	Plasma cell leukemia [Primary refractory]	1 g/m^2, 1 h-iv inf	10000 (90)	0 h (day 1)	1
					1 h (day 1)	NE
					3 h (day 1)	3
					5 h (day 1)	8
					8 h (day 1)	27

Leukemic patients were treated with ara-C, and peripheral blasts were collected at indicated time points. Concentrations of ara-C incorporated into DNA of leukemic cells were quantitated using the present method. AML: acute myeloid leukemia; M2, M5b: subclass of leukemia by French-American-British classification; MDS-LT: leukemic transformation of myelodysplastic syndrome; WBC: peripheral white blood cell; 1 h-iv inf: intravenous infusion for 1 h; 2 h-iv inf: intravenous infusion for 2 h; NE: not evaluated; ND: not detected (below the lower detection limit).

[a] Sampling time points after the end of infusion.

3.3. Monitoring ara-C at the DNA level in the clinic

To confirm clinical utility of the present method, TDM was conducted to determine ara-C incorporation into DNA in vivo in primarily obtained leukemic cells from patients receiving ara-C. Table 3 presents DNA-incorporated ara-C concentrations during intermediate- and high-dose ara-C administration in four leukemic patients. ara-C concentrations varied widely among patients, doses, and sampling time points. Fig. 3A and C presents concentration–time curves in two patients (Nos. 3 and 4), indicating comprehensive TDM of ara-C at the plasma, the cellular, and the DNA levels. To confirm these monitored values, leukemic blasts obtained from the same two patients prior to chemotherapy were incubated in vitro with tritiated ara-C. DNA-incorporated ara-C and intracellular ara-CTP concentrations were measured by scintillation counting (Fig. 3B and D). Concentrations of both metabolites monitored in the clinic (Fig. 3A for patient 3, Fig. 3C for patient 4) were in the same range of those obtained in the culture media with added tritiated ara-C (Fig. 3B for patient 3, Fig. 3D for patient 4), supporting accuracy of this TDM. Thus, the present assay method was capable of measuring ara-C concentration at the DNA level in leukemic cells from patients receiving ara-C chemotherapy. The method provided measurements of intracellular ara-CTP and plasma ara-C concentrations at the same time (Figs. 1 and 3).

4. Discussion

We presently described and validated a sensitive new method for monitoring ara-C concentrations at the DNA level in leukemic cells in vivo. Recovery at each step of the procedure was excellent (Table 1). The method was sensi-

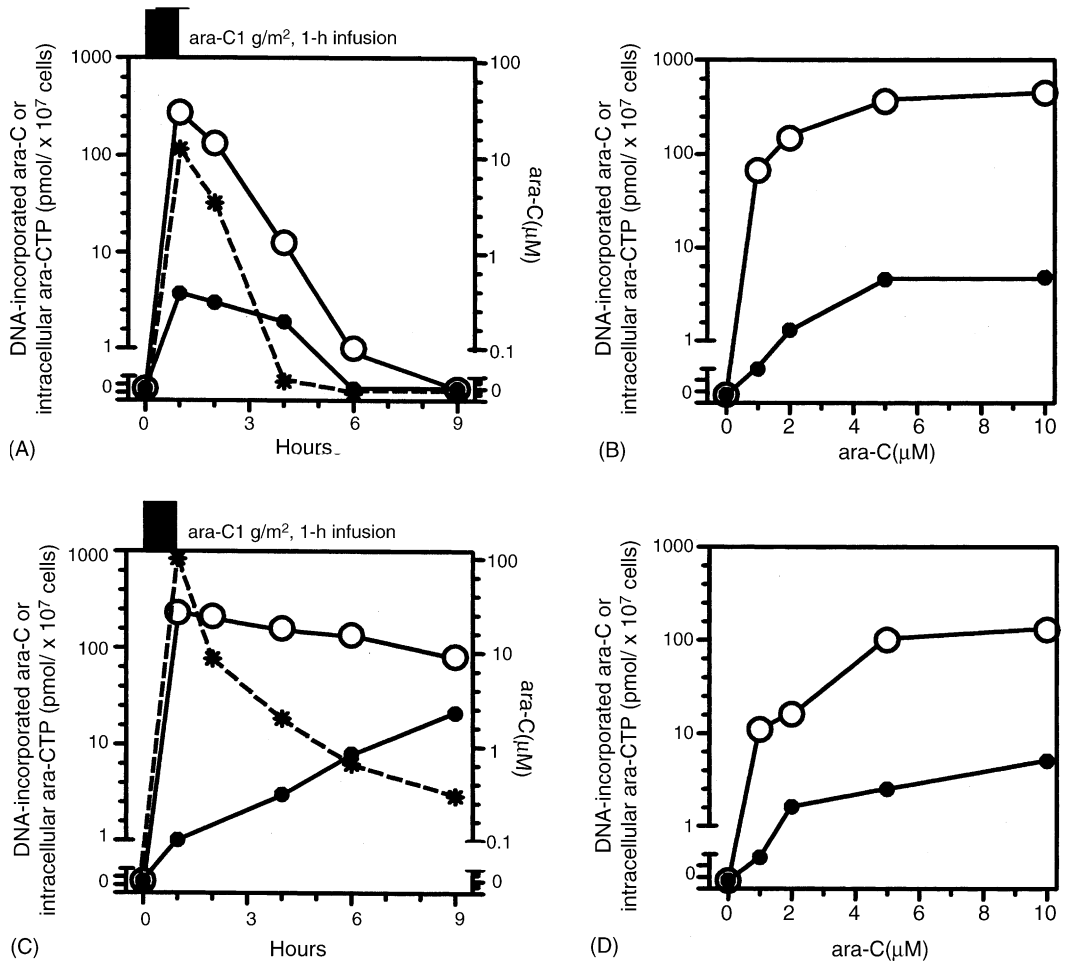

Fig. 3. TDM during ara-C therapy. (A) and (C): Concentration–time curves of plasma ara-C (∗), intracellular ara-CTP (○), and DNA-incorporated ara-C (●). Leukemic patients (A: patient 3, acute myeloid leukemia, M5b, C: patient 4, plasma cell leukemia) were treated with 1 h intravenous infusion of 1 g/m^2 ara-C, and blood samples were collected at the indicated time points (before the treatment, 0 h, 1 h, 3 h, 5 h, and 8 h after the end of the infusion). Concentrations of ara-C incorporated into gemonic DNA of leukemic cells (●) were quantitated using the present method. Concentrations of plasma ara-C (∗) and intracellular ara-CTP of leukemic cells (○) were measured simultaneously. (B) and (D): DNA-incorporated ara-C and intracellular ara-CTP concentrations in pretreatment leukemic cells (B: patient 3; D: patient 4) exposed in vitro to ara-C. Leukemic blasts were obtained prior to chemotherapy from each patient, resuspended in fresh media, and incubated for 6 h with various concentrations (1, 2, 5, and 10 μM) of tritiated ara-C. Intracellular ara-CTP (○) and DNA-incorporated ara-C (●) were measured by scintillation counting.

tive for measuring DNA-incorporated ara-C in cultured leukemic cells treated with 0.5–10 μM ara-C in vitro (Fig. 2). Values obtained were accurate, agreeing with those determined by scintillation counting using tritiated ara-C in parallel experiments (Fig. 2, inset). Precision was confirmed by a minimal interassay variation despite the multistep nature of the process and the use of the biologic materials (Table 2). The method was applied to monitoring DNA-incorporated ara-C concentrations in leukemic blasts from patients being treated with ara-C (Table 3 and Fig. 3). Monitored values were verified by comparison with values determined in the same sample of the patients' cells before treatment using tritiated ara-C in vitro (Fig. 3). The method adapted easily to simultaneous measurement of intracellular ara-CTP and plasma ara-C (Figs. 1 and 3). Thus, the present assay was accurate, precise, and sensitive for clinical monitoring of ara-C concentrations at the DNA level.

A similar method developed by Spriggs et al. for quantitation of ara-C incorporated into DNA of leukemic cells [24] also used DNA fractionation from cells, HPLC separation of ara-C from DNA, and ara-C measurement using radioimmunoassay. The major difference between their method and ours involved DNA fractionation. Spriggs used enzymatic isolation with ethanol precipitation, while we employed the classical STS method. The former was developed simply to isolate intact DNA from the cell, while the latter was developed for quantitation of DNA content in the entire cell [26–28,31]. This suggests that the STS method should achieve better recovery of DNA from the cell than the enzymatic method [32]. Adoption of the STS fractionation should enable our method to obtain more DNA from samples and accurately measure lower concentrations of DNA-incorporated ara-C. Our method also may be better suited to simultaneous measurement of intracellular ara-CTP and plasma ara-C concentrations. Thus, our method was used to monitor drug concentrations at the DNA level over time during ara-C therapy; in contrast, no data obtained using the previous method have been reported since the initial study.

The present assay method monitored ara-C concentrations at the DNA level in circulating blasts from leukemic patients receiving ara-C (Table 3, Fig. 3A and C). In patient 3 (Fig. 3A), concentrations of DNA-incorporated ara-C, intracellular ara-CTP, and plasma ara-C were greatest at the end of the infusion. All three forms of ara-C decreased gradually, and fell below the quantitation limit by 8 h after the infusion. Elimination of DNA-incorporated ara-C appeared to be slower than that of intracellular ara-CTP, in agreement with previous in vitro results in which DNA-incorporated ara-C was more persistent than intracellular ara-CTP [13,33,34]. In patient 4 (Fig. 3C), both plasma ara-C and intracellular ara-CTP were maintained longer than in patient 3. This appears to reflect our previous finding that maintenance of plasma ara-C was necessary for retention of ara-CTP in leukemic cells [22,23]. Unlike that in patient 3, the DNA-incorporated ara-C concentration in patient 4 was even increased between 5 and 8 h after the end of the infusion (Fig. 3C). This might be attributed to prolonged retention of ara-CTP in cells, since ara-C incorporation into DNA requires intracellular ara-CTP formation [35,36]. Moreover, as maintenance of plasma ara-C is necessary for ara-CTP formation [22,23], relatively slow elimination of plasma ara-C in patient 4 might be primarily responsible for prolonged incorporation of ara-C into DNA. Thus, DNA incorporation of ara-C might depend in part on intracellular retention of ara-CTP or persistence of plasma ara-C.

Intracellular ara-CTP concentrations in patient 3 (Fig. 3B) were similar to those determined in cultured HL 60 cells (30–300 pmol/1 $\times 10^7$ cells) [34]. However, ara-C concentrations at the DNA level in patient 3 (1–10 pmol/1 $\times 10^7$ cells, Fig. 3B) were much lower than those of HL 60 cells (10–80 pmol/1 $\times 10^7$ cells, Fig. 2A). Because the extent of ara-C incorporation into DNA is cell-cycle dependent [7,8,11,12,35], greater ara-C incorporation into DNA of cultured cells may reflect a larger S-phase fraction than that in patient's circulating blasts. In relapsed leukemia, various mechanisms of ara-C resistance such as the increases in DNA polymerases [36] and in DNA exonuclease activity [37,38] also may reduce DNA incorporation of the drug despite intracellular retention of ara-CTP. If so, the ara-C concentration at the DNA level may not always be proportional to the intracellular ara-CTP concentration and therefore not be entirely predictable from plasma or cellular pharmacokinetics. Moreover, the DNA-incorporated ara-C concentration in patient 4 increased between 5 and 8 h after ara-C infusion while no further incorporation was observed at 5 h in patient 3 (Fig. 3A and C). In addition to the pharmacokinetic consideration above, the ara-C concentration at the DNA level in patient 4 may not have been fully sufficient to terminate DNA chain elongation, thus allowing the drug to be incorporated further into the blasts' replicating DNA. This also suggested a possibility that individual patients may have different target drug concentrations at the DNA level for maximal inhibition of DNA synthesis. Accordingly, DNA-incorporated ara-C may be worthy of pharmacokinetic evaluation as an independent parameter for prediction of therapeutic outcome with ara-C.

A combination of regular-dose ara-C given for 7 days with daunorubicin given for 3 days has been a standard induction therapy for the last 20 years. This regimen achieves complete remission rates of over 70% in adult patients with acute myeloid leukemia [1–3]. However, the remissions are not durable, and long-term survivors account for only 30–40% of patients. Because achievement of complete remission is indispensable for long-term survival and cure, reinforcement of induction therapy may improve results in terms of both rate and quality of the remission. Quality or depth of remission may differ among patients responding to the same induction regimen because

their leukemic cells vary in drug sensitivity. As ara-C is the key drug in remission induction therapy, quality of remission well may depend on sensitivity of the cells to ara-C. Therapeutic outcome with ara-C has been closely associated with intracellular pharmacokinetics as opposed to the plasma ara-C concentration [15–20,39,40]. However, ara-C-induced cytotoxicity in vitro has been found to be determined primarily by the extent of incorporation of ara-C into DNA [13]. If this also is the case in the clinical setting, ara-C therapy may be individualized best according to the pharmacokinetics not only of intracellular ara-CTP but also of DNA-incorporated ara-C. We have developed sensitive techniques for clinically monitoring intracellular ara-CTP [21] and now also DNA-incorporated ara-C (Fig. 1). Therapy for leukemia thus may be individualized based on TDM at the DNA level for ara-C.

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