Dephosphorylation of Nucleotides of 1- β -D-Arabinofuranosylcytosine in Relation to the Different Drug Sensitivity in Tumor Cells

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ABE, I. and SATO, H. *Dephosphorylation of Nucleotides of 1- β -D-Arabinofuranosylcytosine in Relation to the Different Drug Sensitivity in Tumor Cells.* Tohoku J. exp. Med., 1979, 127 (3), 281-288 — Dephosphorylation of nucleotides of 1- β -D-arabinofuranosylcytosine (ara-C) was studied to clarify the intracellular metabolism of ara-C and the mechanism of natural resistance in the rat ascites hepatomas. The apparent rate of phosphorylation of ara-C in the intact cells was the balance of the true phosphorylation and the dephosphorylation of nucleotides formed, which was visible after the cessation of the phosphorylation. Ara-C nucleotides formed in the intact cells degraded upon the addition of iodoacetate as a result of ATP depletion in the cells thereby interfering with the ara-C phosphorylation and some additional mechanisms. Decrease in ara-C nucleotides after the dilution of ^3H -ara-C with unlabeled ara-C was similar to that after the washing-out of the substrate, and longer than after the addition of iodoacetate. Half-life of ara-C nucleotides was shorter in the more resistant tumors; i.e., the dephosphorylation of the nucleotides was active in AH109A, AH60C, and AH66F in the decreasing order in accord reciprocally with the intracellular level of ara-C nucleotides and their drug sensitivity. Therefore, the rate of the nucleotide dephosphorylation is important together with the phosphorylation of ara-C in the maintenance of the active form of the drug, ara-C triphosphate. — ara-C nucleotides; dephosphorylation; drug resistance; rat ascites hepatomas

In the previous paper (Abe et al. 1978a), where the capacity for the phosphorylation of 1- β -D-arabinofuranosylcytosine (ara-C) was reported to be important in the mechanism of resistance of the rat ascites hepatomas to the drug, the dephosphorylation of ara-C nucleotides subsequent to the phosphorylation was described to occur in the intact hepatoma cells after the addition of iodoacetate. The significance of the dephosphorylation of the nucleotides has not been clarified yet, although the mechanism of resistance to ara-C has been investigated in human and experimental tumors (Chu and Fischer 1965; Creasey et al. 1966; Momparler et al. 1968; Uchida and Kreis 1968; Kessel et al. 1969;

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Drahovsky and Kreis 1970; Schrecker 1970; Steuart and Burke 1971; Kreis et al. 1972; Nakamura et al. 1972; Chou et al. 1977).

MATERIALS AND METHODS

Ara-C was kindly supplied from Nippon Shin-yaku Co., Kyoto. ^3H -Ara-C was purchased from the Radiochemical Centre, Amersham, England. Other chemicals were of the reagent grade. Rat ascites hepatomas, AH66F, AH60C and AH109A, and mouse leukemia L1210 cells were collected by peritoneal lavage with 1 mM EDTA-saline (pH 7.4).

Incubation of the intact cells with ^3H -ara-C was performed by the slight modification (Abe et al. 1978a) of the method by Schrecker and Urshel (1968). In brief, the cell suspension (0.05 g packed cells per ml) was incubated in Ca^{++} free Krebs-Ringer phosphate (pH 7.4) supplemented with 1 mM glucose (KRP), and 0.02 mM ^3H -ara-C, unless otherwise indicated. For the observation of the dephosphorylation, the phosphorylation of ara-C was stopped by either the addition of 5 mM iodoacetate, the dilution of ^3H -ara-C with 1 mM unlabeled ara-C (50-fold concentration), or the washing-out of ^3H -ara-C by the 3 cycles of alternate dilution of the suspension with cold KRP and centrifugation. Activity of the cytoplasmic extracts to phosphorylate ara-C was examined in the reaction mixture (Abe et al. 1978b) consisting of ^3H -ara-C, the concentration of which was given in Figure and Table, 0.05 M Tris-HCl (pH 8.0), 5 mM ATP, 3 mM MgCl_2 , and 0.6 mg/ml of protein of the supernatant fraction of AH66F cells (1 g packed cells yielding 48 mg protein in the soluble fraction after the centrifugation at $30,000 \times g$, 60 min) according to Momparler and Fischer (1968), and de Saint-Vincent and Buttin (1973). After the incubation at 37°C for the indicated period, aliquots were applied to DE-81 paper discs for the determination of ara-C nucleotides. ATP concentration was determined by the method of Estabrook et al. (1967).

RESULTS

Phosphorylation of ara-C progressed linearly within, at least, 20 min, and fell off thereafter first in AH109A, and then in AH60C and AH66F under the untreated conditions (Figs. 1 and 2). By addition of iodoacetate, the phosphorylation of ara-C was stopped and the dephosphorylation started in the rat ascites hepatomas and bone marrow cells (Fig. 1) similarly to the previous report using the higher substrate concentration (Abe et al. 1978a). Radioactivity of arabinonucleotides decreased to the background level within around 30 min in all the cells tested. If the dephosphorylation of ara-C nucleotides observed after the addition of iodoacetate is of physiological significance in the metabolism of ara-C, then the dephosphorylation may not be related to the difference in the drug sensitivity among the 3 ascites hepatomas because the dephosphorylation was equally strong in those cells irrespective of their drug sensitivity. By contraries, the dephosphorylation of ^3H -ara-C nucleotides after the 50-fold dilution of ^3H -ara-C with unlabeled ara-C was not so rapid as after the addition of iodoacetate (Fig. 2). In addition, the rate of dephosphorylation of the nucleotides differed from tumor to tumor as mentioned later. Therefore, the effect of iodoacetate on the ara-C metabolism was further investigated. Iodoacetate inhibited ara-C kinase only slightly, when examined in the cell-free system (Table 1, System I). KF apparently stimulated the phosphorylation of ara-C, probably by inhibiting the

Fig. 1. Effect of iodoacetate on the intracellular level of ara-C nucleotides in the intact AH66F, AH60C, AH109A and rat bone marrow cells.

Cell suspension (0.05 g/ml) in KRPG containing 0.02 mM ^3H -ara-C (0.25 Ci/mmole) was incubated at 37°C. 50 μl aliquots were removed at every 10 min and applied to DE-81 paper discs. The discs were washed by 3 cycles of alternate immersion in 1 mM HCOONH_4 and water, dried and subjected to liquid scintillation counting for radioactivity. $\circ$, control; $\bullet$, iodoacetate (5 mM in final) was added 20 min after the onset of incubation ($\downarrow$).

Fig. 2. Effect of the dilution of ^3H -ara-C with 50-fold concentration of unlabeled ara-C on the intracellular level of ara-C nucleotides in the intact AH66F, AH60C, AH109A and L1210 cells.

Experimental design was similar to Fig. 1. $\circ$, control; $\bullet$, unlabeled (cold) ara-C was added to 1 mM in final (50-fold concentration) 30 min after the onset of incubation ($\downarrow$).

dephosphorylation. When the phosphate donor was replaced by ATP generating system (System II) instead of ATP itself, iodoacetate inhibited the phosphorylation of ara-C. KF also inhibited the phosphorylation to a lesser extent. Optimal concentration of ATP was about 5 mM, and half the maximum activity was obtained at 1.4 mM in the cell-free system (Fig. 3). ATP concentration in the

TABLE 1. Effect of iodoacetate on the phosphorylation of ara-C in AH66F

		Arabinonucleotides formed (cpm/sample/80 min)
System I*	Complete	1,724
	- ATP	45
	+ Iodoacetate 5mM	1,499
	+ KF 9mM	1,966
System II†	Complete	1,729
	- ADP, FDP, NAD ⁺	25
	+ Iodoacetate 5 mM	209
	+ KF 9mM	893

* The phosphate donor was ATP in the System I. The incubation medium consisted of 0.1 mM ³H-ara-C (0.25 Ci/mMole) 5mM ATP, 3mM MgCl₂, 0.6 mg protein of the cytoplasmic extract/ml, 0.05M Tris-HCl pH (8.0).

† ATP generating system was used instead of ATP itself. ATP was replaced by 2mM ADP, 5 mM fructose-1,6-diphosphate, 0.5 mM NAD⁺, 1mM Pi. Glycolytic enzymes were supplied intrinsically by the cytoplasmic extract.

Fig. 3. Effect of ATP concentration on ara-C kinase activity in the cytoplasmic extract of AH66F cells.

Reaction mixture consisted of 0.5 mM ³H-ara-C (0.1 Ci/mMole), 1–10 mM ATP, 3 mM MgCl₂, 9 mM KF, 2.5 mM mercaptoethanol, 0.05 M Tris-HCl (pH 8.0), and 2.4 mg protein of 30,000 × g extract of AH66F cells/ml. Reaction progressed linearly over 120 min. 50 μl aliquots were applied to DE-81 paper discs for determination of radioactivity of ³H-ara-C nucleotides adsorbed.

intact AH66F cells was 3.3 mM and decreased to 1 mM 4 min after the addition of iodoacetate (Fig. 4), when the dephosphorylation was in the midst of the process (Fig. 1). Similar effect of iodoacetate on ATP concentration was reported in Novikoff hepatoma cells (Plagemann et al. 1976).

The intracellular level of ara-C nucleotides after 60 min was higher in AH60C than in AH109A, although the initial rate of the phosphorylation was similar between them, and proportional to the half-life of the nucleotides in these rat ascites hepatomas. Half-lives of ara-C nucleotides determined by the dilution of

Fig. 4. Effect of iodoacetate on the intracellular level of ATP in AH66F cells.

AH66F cells were collected 4 days after the intraperitoneal inoculation, washed and suspended in KRPG (0.05 g/ml). After preincubation for 5 min, iodoacetate was added to 5 mM in final (0 time). ○, control; ●, iodoacetate added. At each time interval, a 2 ml aliquot was removed, flashed into ice-cold saline and centrifuged at $1,000 \times g$ for 30 sec. Pellet was treated with 6% ice-cold perchloric acid, stood for 10 min and centrifuged. Acid extract was removed and neutralized with 6 M K_2CO_3 and centrifuged. ATP concentration in the extract was determined by spectrophotometry of NADP reduction coupling with ATP consumption and glycolysis according to Estabrook et al. (1967). Every one molecule of ATP is consumed as a phosphate donor, one molecule of $NADP^+$ is reduced to NADPH, during the chain reaction of glucose to 6-phosphoglucono- δ -lactone through glucose-6-phosphate in the coupled enzyme system of hexokinase and glucose-6-phosphate dehydrogenase. Reaction mixture containing 0.2 mM $NADP^+$, 50 mM triethanolamine-HCl (pH 7.4), 10 mM KCl, 5 mM EDTA, 10 mM $MgCl_2$, 10 mM glucose, 2 U/ml glucose-6-dehydrogenase and acid extract was transferred to the absorption tube and the reaction was started by adding hexokinase (2 U/ml). Reduction of $NADP^+$ to NADPH was monitored by increase in absorption at 340 nm. Average extinction coefficient for NADPH at 340 nm was assumed to be $6.3 \times 10^3 \text{ cm}^{-1} \cdot \text{M}^{-1}$.

Each point and vertical bar are mean and s.d. of 3 experiments.

^3H -ara-C with unlabeled ara-C were quite similar to those by the washig-out of substrate, and 150 min, 68 min, and 42 min in AH66F, AH60C, and AH109A respectively (Table 2) in accord with their drug sensitivity (Abe et al. 1978c). Half-life of ara-C nucleotides in L1210 was similar to the reported value being about 50 min (Momparker 1973). Because L1210, which is sensitive to ara-C (Evans et al. 1964; Dixon and Adamson 1965; Wodinsky and Kensler 1965; Kline et al. 1966), had a high capacity for phosphorylating ara-C, the intracellular level of ara-C nucleotides was higher in L1210 than in the hepatomas in spite of the same half-life of the nucleotides in L1210 as that in the AH60C cells. Half-life of the nucleotides after the addition of iodoacetate was much shorter than 30 min (Fig. 1) but not estimated because the process of the dephosphorylation did not follow the first-order kinetics nor might be physiological as discussed later.

TABLE 2. Intracellular concentration and half-life of ^3H -ara-C nucleotides

	^3H -Ara-C nucleotides* (nmoles/g cells)		Half-life (min)	
	At 5 min	At 60 min	Dilution†	Washing-out‡
AH66F	7.2±1.9(8)	75.1±11.7(5)	150	127
AH60C	3.2±0.6(6)	31.6± 4.8(5)	68	64
AH109A	3.1±0.2(6)	18.8± 2.2(5)	42	50
L1210	49.1±5.9(2)	380.3±58.2(3)	64	—

* Intracellular concentration of ^3H -ara-C nucleotides formed during the incubation for the first 5 min and 60 min respectively. Ara-C concentration, 0.1 mM. Cell density, 0.05 g packed cells/ml of KRPG. The number of the experiments is shown in parenthesis.

† Dephosphorylation of ^3H -ara-C nucleotides was visible after the apparent cessation of the phosphorylation by the dilution of ^3H -ara-C with 50-fold concentration of unlabeled ara-C. Half-lives listed are means of the two experiments.

‡ Phosphorylation of ara-C was stopped by the washing-out of ^3H -ara-C. See Materials and Methods section for detail.

DISCUSSION

The dephosphorylation of preformed ara-C nucleotides was suggested to be important in the metabolism of ara-C in the rat ascites hepatomas. Effect of iodoacetate on the intracellular metabolism of ara-C was remarkable (Fig. 1). Ara-C kinase itself was resistant to iodoacetate in AH66F cells (Table 1), AH60C and AH109A (not shown). The enzyme activity of L1210 was reported to be also resistant to iodoacetate (Kessel 1968). Inhibition of the phosphorylation of ara-C by iodoacetate may be due partly to inhibition of ATP generation (Table 2). Glycolysis has been well known to be highly susceptible to iodoacetate (Webb 1966). However, the decrease in the intracellular level of ATP was not so rapid (Fig. 4) considering the very rapid cessation of the phosphorylation and the onset of the dephosphorylation (Fig. 1). Intracellular ATP remaining after the addition of iodoacetate might be restricted within some compartment so that it was not utilized to the ara-C phosphorylation. Another possibility may be that the dephosphorylation was accelerated by iodoacetate; the activation of phosphatase or the solubilization of phosphatase(s) compartmented under the physiological conditions. That the degradation started after the initial lag in AH60C and bone marrow cells might prefer the latter possibilities. So far, no evidence on this line has been obtained yet. On the contrary, the dephosphorylation of ara-C nucleotides after the dilution of ^3H -ara-C with unlabeled ara-C or the washing-out of the labeled substrate was in good accordance (Table 2).

The intracellular level of the nucleotides, mostly consisting of ara-C triphosphate, after the 60 min (Table 2) or 80 min (Fig. 2) incubation was not merely a reflect of the capacity for the ara-C phosphorylation but significantly affected with the extent of the nucleotide dephosphorylation. Incubation for more than 80 min was not attempted because the experimental conditions might deviate from the physiological

state. That the intracellular level of ara-C nucleotides after 60-min incubation would not be artefact may be supported with the fact that the nucleotide level was proportional not only to the rate of dephosphorylation but also to the drug sensitivity.

In conclusion, the nucleotide dephosphorylation was clarified to be important in the drug sensitivity in the rat ascites hepatomas as well as the phosphorylation of ara-C. Effect of ara-C triphosphate, when given as a liposome-encapsulated form, might depend on the capacity for the dephosphorylation in the tumor cells.

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