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Activation of 5′-Deoxy-5-fluorouridine by Thymidine Phosphorylase in Human Tumors

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Activities of pyrimidine nucleoside phosphorylases were assayed in extracts of human tumors, normal tissues of the same organs and tumors of mice (Sarcoma-180) and guinea pigs (Line-10), with thymidine (dThd), uridine (Urd), and 5′-deoxy-5-fluorouridine (5′-DFUR) as substrates. The nucleoside cleaving activities were higher in extracts of human tumor tissues than in those of normal tissues of the same organs. In human tissues, phosphorolytic activitiy towards dThd was high, while that towards Urd was low. In animal tumors, Urd was the best substrate. 1-(2′-Deoxy-β-D-glucopyranosyl)-thymine (GPT), a specific inhibitor of uridine phosphorylase, inhibited the phosphorolysis of Urd and 5′-DFUR in extracts of animal tumors, but not that of dThd and 5′-DFUR in extracts of human tumors. A thymidine phosphorylase preparation was partialy purified from human lung cancer. K_m values of the preparation were 2.43×10^{-4} M and 1.69×10^{-3} M for dThd and 5′-DFUR, respectively. We conclude that in human tumors a thymidine phosphorylase activity converts 5′-DFUR to 5-fluorouracil, an activated form.

Keywords——5′-deoxy-5-fluorouridine; thymidine; uridine; 5-fluorouracil; 1-(2′-deoxy-β-D-glucopyranosyl)-thymine; thymidine phosphorylase; uridine phosphorylase; human tumor; lung cancer

5′-Deoxy-5-fluorouridine (5′-DFUR) is an antitumor agent recently synthesized by Cook *et al.*[1] Experimental studies have shown that it has a broad antitumor spectrum and is less toxic than other fluorinated pyrimidines.[1-3] 5′-DFUR is a chemical depot form of 5-fluorouracil (FUra).[2] The antitumor activity of 5′-DFUR is manifested after its cleavage to FUra, as is postulated for other FUra derivatives.[4,5] The cleavage is catalyzed by pyrimidine nucleoside phosphorylases.

There are two distinct pyrimidine nucleoside phosphorylases. One is thymidine phosphorylase (EC 2.4.2.4), which catalyzes the phosphorolysis of thymidine and is reported to be specific for 2′-deoxyribonucleosides.[6,7] The other is uridine phosphorylase (EC 2.4.2.3); this phosphorylase acts primarily on uridine, though a broad substrate specificity has been reported.[8] Recently, the existence of a third pyrimidine nucleoside phosphorylase activity, uridine-deoxyuridine phosphorylase, was suggested.[9]

In experimental tumors of animals, the activation of 5′-DFUR is catalyzed by uridine phosphorylases.[2a] We examined the enzymatic cleavage of 5′-DFUR in human tumors and concluded that thymidine phosphorylase plays an important role in this respect.

Experimental

Materials——All chemicals were of analytical grade and were purchased from Wako Pure Chemical Co., Osaka, unless otherwise stated. 5′-DFUR was provided by Hoffmann-La Roche Inc., Nutley, N.J., U.S.A. 1-(2′-Deoxy-β-D-glucopyranosyl)-thymine (GPT) (NSC 402666) was obtained from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, U.S.A.

Tissue Extracts——Human tumor and normal tissues of the same organs obtained at operations were stored frozen (−20°C). Tumor and normal tissues were separated and examined histologically. Tissues were

homogenized in approximately 10 volumes of 10 mM Tris buffer (pH 7.4) containing 15 mM NaCl and 1.5 mM $MgCl_2$. The homogenates were centrifuged at 105000×*g* for 90 min at 4°C and the supernatants were dialyzed overnight against 500 volumes of 20 mM phosphate buffer (pH 7.4). The clear solution thus obtained was used immediately as tissue extract.

Sarcoma-180 (5×10^7 cells) and Line-10 (5×10^7 cells) were subcutaneously transplanted into IRC mice (body weight, *ca.* 25 g) and strain 2 guinea pigs (body weight, *ca.* 250 g), respectively. After 3 weeks, solid tumors were excised and tissue extracts were obtained by a procedure similar to that used for human tissues. These extracts were subjected to enzyme assay immediately after preparation.

Pyrimidine Nucleoside Phosphorylase Activity——In a 20-ml test tube, 0.2 ml of aqueous solution of a substrate (16 nmol pyrimidine nucleoside/0.2 ml), 0.2 ml of 0.36 M phosphate buffer (pH 7.4), 0.2 ml of 0.49 M NaCl, and 0.2 ml of 12 mM KCl were mixed. The mixture was preincubated at 37°C for 5 min. Then 0.2 ml of an aqueous solution of the tissue extract was added and the whole was incubated at 37°C for 5—60 min.

Linear reaction kinetics were maintained for the period of the measurement. The reaction was terminated by addition of 8 ml of cold ethyl acetate. The organic layer was separated after shaking of the mixture and concentrated *in vacuo*. The residue was dissolved in methanol.

A portion of the methanol solution was injected into a Waters liquid chromatograph equipped with an ultraviolet (UV) detector operated at 254 nm. A μ-Bondapak C_{18}/Porasil column was used and the mobile phase was water. Thymine, FUra, and uracil each showed a single sharp chromatographic peak, and they were determined from the ratios of their peak heights to that of an internal standard. The internal standards were 5-chlorouracil for thymine and FUra and thymine for uracil.[10]

Inhibition studies were carried out in the presence of 0 to 0.5 mM GPT. Protein concentrations in the preparations were determined by the method of Lowry *et al.*[11]

Partial Purification of the Enzyme——Human lung tumor tissues (poorly differentiated adenocarcinoma from a 62-year-old male) were washed in ice-cold 0.9% NaCl solution and homogenized in 10 volumes of 10 mM Tris buffer (pH 7.4) containig 15 mM NaCl and 1.5 mM $MgCl_2$. The homogenates were centrifuged at 7000×*g* for 30 min at 4°C and the supernatant was treated with ammonium sulfate. The fractions obtained between 20 and 40% saturation were dialyzed against 20 mM phosphate buffer (NaH_2PO_4–Na_2HPO_4; pH 7.4) containing 10 mM β-mercaptoethanol. The protein solution thus obtained was purified through chromatography on a ConA Sepharose column (2.1×8 cm) eluted with the same buffer as used in the dialysis. The effluents were further subjected to chromatography on a column of diethylaminoethyl (DEAE) Sephacel (1.5×15 cm). The column was developed with 20 mM phosphate buffer (KH_2PO_4–K_2HPO_4; pH 7.5) containig 10 mM β-mercaptoethanol and a linear gradient of KCl (0—400 mM). The fractions with the highest dThd cleaving activity (70—130 mM KCl) were collected.

Results

The activities for phosphorolysis of dThd, Urd, and 5′-DFUR were measured in extracts from human tumors and normal tissues of the same organs. The results are listed in Table I. Higher phosphorylase activities were noted in tumors than in normal tissues. The results for tumors of mice (Sarcoma-180) and guinea pigs (Line-10) are tabulated in Table II.

There was a large difference in phosphorolytic activities between human and animal tumors. In extracts of human tissues, activities for cleaving the three pyrimidine nucleosides were in the order of dThd, 5′-DFUR, and Urd. On the other hand, extracts of animal tumors cleaved Urd more rapidly than dThd and 5′-DFUR. The phosphorolytic activity for dThd and for 5′-DFUR was quite low in the extract of the guinea pig tumor.

GPT reportedly inhibits uridine phosphorylase activity, but does not inhibit the activity of thymidine phosphorylases.[9,12,13] In the presence of GPT, phosphorolysis of Urd was inhibited in extracts of tumors of humans, mice, and guinea pigs (Tables II and III). GPT inhibited the phosphorolysis of 5′-DFUR in the extract of mice tumors. With the extracts of human tumors, the cleavages of dThd and 5′-DFUR were unaffected by GPT.

From studies of several animal tumors, evidence has been reported to suggest that uridine phosphorylase catalyzes the phosphorolysis of 5′-DFUR.[2] The present results suggest that in human tissues thymidine phosphorylase activity is responsible for the phosphorolysis of 5′-DFUR.

In order to obtain further information, purification of thymidine phosphorylase from human lung tumor was undertaken. The specific activity of a partially purified preparation represents a 400-fold purification of the initial homogenate. The results of kinetic analyses

with this preparation were in agreement with Michaelis-Menten kinetics with dThd and 5'-DFUR as substrates. K_m values were 2.43×10^{-4} M and 1.69×10^{-3} M for dThd and 5'-DFUR, respectively (substrate concentration, 4×10^{-5}—1×10^{-2} M). Further purification of the enzyme is in progress in our laboratories.

TABLE I. Pyrimidine Nucleoside Phosphorolytic Activities of Human Tumor and Normal Tissues of the Same Organ

Organ	Tissue	Substrate[a]		
		dThd	Urd	5'-DFUR
Stomach	Tumor[b]	1150± 1.6	10.8±0.0	40.6± 0.2
	Normal	381±10.4	3.6±0.0	13.4± 0.2
Stomach	Tumor[c]	674± 1.6	14.8±0.6	56.0± 0.4
	Normal	64± 1.0	3.0±0.0	4.4± 0.0
Lung	Tumor[d]	2132±17.8	60.4±0.0	148.0± 5.2
	Normal	93± 5.0	4.6±0.0	9.2± 0.4
Lung	Tumor[e]	2210±46	36.4±0.6	161.0±11.6
	Normal	105+ 6.8	3.8+0.2	9.6+ 0.4

a) The nucleosides (16 nmol) were incubated (5—30 min, 37 ℃) with extracts of tissues (protein content, 15—30 μg) in 72 mM phosphate buffer (pH 7.4). Values are nmol pyrimidines formed by 1 mg proteirn in 1 h. Mean ± range of 2 determinations.
b) Well differentiated adenocarcinoma from 56-year-old male.
c) Poorly differentiated adenocarcinoma from 45-year-old male.
d) Anaplastic large cell carcinoma from 48-year-old male.
e) Well differentiated squamous cell carcinoma from 71-year-old male.

TABLE II. Pyrimidine Nucleoside Phosphorolytic Activities in Animal Tumors[a]

Animal tumors	Substrate	Without GPT	With GPT
Sarcoma-180	dThd	4.52±0.51	0.65±0.08
	Urd	38.3 ±1.7	10.2 ±1.1
	5'-DFUR	2.98±0.33	0.28±0.08
Line-10	dThd	0.91±0.26	0.45±0.31
	Urd	56.8 ±5.3	2.71±0.71
	5'-DFUR	0.62±0.01	Not detectable

a) The nucleosides (16 nmol) were incubated (1 h, 37 ℃) with the extracts (protein, 300—600 μg) in 72 mM phosphate buffer (pH 7.4) in the absence or presence of GPT (73.5 nmol). Values are nmol pyrimidines formed by 1 mg protein in 1 h and are the means ± SD of 4 experiments.

TABLE III. Pyrimidine Nucleoside Phosphorolytic Activities of Extract of Human Lung Tumor in the Presence and Absence of GPT[a]

Substrate	Without GPT	With GPT
dThd	42.8±0.8	60.0 ±1.8
Urd	13.3±0.2	1.97±0.15
5'-DFUR	24.2±0.1	26.1 ±1.7

a) The nucleosides (16 nmol) were incubated (1 h, 37°C) with extract of human lung tumor (moderatly differentiated papillary adenocarcinnma from 59-year-old male) in 72 mM phosphate buffer (pH 7.4) in the absence or presence of GPT (147 nmol). Values are nmol pyrimidines formed by 1 mg protein in 1 h, and are the means ± SD of 5 determinations.

Discussion

The results shown in Table I indicate that pyrimidine nucleoside phosphorylase activities are considerably higher in tumor tissues than in normal tissues of the same human organ. GPT is a specific inhibitor of uridine phosphorylase activity.[12] GPT inhibited the phosphorolysis of Urd, but not that of dThd or 5'-DFUR by the extracts of human tumors. Since GPT does not inhibit thymidine phosphorylase activity, the results indicate that 5'-DFUR was cleaved by a thymidine phosphorylase activity. This was confirmed by the results obtained with the partially purified thymidine phosphorylase preparation from human lung cancer. Our recent experimental results, which will be reported shortly, suggest that the substrate specificity of the human thymidine phosphorylase is broader than it has been reported to be.[6,7]

The results for animal tumors in Table II indicate that uridine phosphorylase activity was much higher than thymidine phosphorylase activity. In mouse tumors, the cleavage of 5'-DFUR is catalyzed by uridine phosphorylase. This is in agreement with the results of the previous paper.[2a]

In the analyses of antitumor activities of 5'-DFUR and its analogs, special consideration may be necessary of the differences in pyrimidine phosphorylase activities between human and animal tumors.

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