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ENZYMES OF SALVAGE AND *DE NOVO* PATHWAYS OF SYNTHESIS OF PYRIMIDINE NUCLEOTIDES IN HUMAN COLORECTAL ADENOCARCINOMAS

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Abstract—The activity of uridine–cytidine kinase (Urd–Cyd kinase), a key enzyme in the salvage of pyrimidine nucleosides, averaged 0.86 ± 0.16 (S.E.M.) nmole uridine phosphates·min^{-1}·(mg protein)$^{-1}$ in fifty-three specimens of human colorectal adenocarcinomas. The activity of fluorouracil phosphoribosyltransferase (FUPRTase) in thirty-five carcinoma specimens averaged only 0.19 ± 0.07 nmole fluorouridine phosphates·min^{-1}·(mg protein)$^{-1}$. The activity of the last enzyme in the *de novo* pathway of biosynthesis of UMP, i.e. orotidine 5′-monophosphate (OMP) decarboxylase, averaged 0.21 ± 0.04 nmole CO_2·min^{-1}·(mg protein)$^{-1}$. The activity of Urd–Cyd kinase was increased ~2.3-fold, and that of OMP decarboxylase by about 91%, while that of FUPRTase was increased by only 27%, as compared to that of normal human colonic mucosa. Of the colorectal carcinomas studied, 72% were moderately differentiated, 21% poorly differentiated, and 7% well differentiated. The mean diameter of the fifty-three carcinomas was 5.5 cm, and pathologic staging led to classification of 15% as Dukes' A, 36% as Dukes' B, 47% as Dukes' C, and 2% as carcinoma *in situ*. No correlations between the level of the enzyme activities studied and any pathologic characteristics of the carcinomas could be discerned.

Colorectal adenocarcinomas represent a major cause of cancer death [1, 2] in humans, and only temporary remissions have been achieved with currently employed chemotherapeutic agents [3]. Urd–Cyd kinase (EC 2.7.1.48) catalyzes the formation of the corresponding 5′-monophosphate esters (i.e. UMP and CMP), which eventually are converted to the di- and triphosphates. This reaction is rate-limiting in the salvage of Urd and Cyd [4, 5]. Study of these enzymes in human colorectal adenocarcinomas and of the balance between these two alternative pathways (*de novo* and salvage) may give clues as to more appropriate uses of available chemotherapeutic agents in this presently drug-refractory neoplasm, while new and better agents are being sought. In colorectal adenocarcinomas, little information is available concerning the relative efficacy of the enzymes involved in (a) the salvage of uracil, Urd and Cyd and (b) the biosynthesis *de novo* of UMP (from which a large number of derivatives of UMP and CMP, including RNA and DNA, are formed). Our earlier studies were focused on the activity of Urd–Cyd kinase, found in a variety of solid and ascites tumors [6–8], and we have reported previously on the activity of Urd–Cyd kinase in twenty-two specimens of human colorectal adenocarcinomas [9]. In the present study, however, fifty-three specimens of these human neoplasms have been examined, as have the interrelationships between the two salvage enzymes (Urd–Cyd kinase and FUPRTase), while the activity of the enzyme involved in the last step in the *de novo* pathway for the synthesis of UMP (i.e. OMP decarboxylase; EC 4.1.1.23) has been investigated. A recent report [10], related to these studies, included findings obtained with nine human colon carcinomas.

MATERIALS AND METHODS

Specimens of primary human colorectal neoplasms, as described previously [9], were obtained as soon as possible after surgery, homogenized and centrifuged (100,000 *g*), and the supernatant fraction was brought to 30% saturation with ammonium sulfate. After the removal of the precipitate by centrifugation, the level of saturation with ammonium sulfate was increased to 50%, and the resultant precipitate was removed by centrifugation and twice dialyzed, as previously described [9]. Urd–Cyd kinase activity was assessed under optimal conditions and with linear kinetics [9], making use of the assay employing DEAE-cellulose discs developed by one of us [11]. The incubation mixture contained in a final volume of 200 μl: Tris–HCl buffer (pH 7.5–7.8), 200 mM; β-mercaptoethanol, 10.0 mM; $MgCl_2$, 12.5 mM; ATP, 13.9 mM; [5-^{3}H]uridine (0.25 μCi), 10.0 mM; and 600–900 μg of protein, derived from the dialyzed ammonium sulfate fraction. After agitation in a water bath (37–40°) for 40 min, the reaction was stopped by immersion of the tubes for 3 min in boiling water. Denatured protein was removed by centrifugation and the samples were processed as described previously [6, 11].

FUPRTase activity was determined in the cytosolic fraction of tissues (clarified at 100,000 *g*). The incubation was carried out at 37° for 15 and 30 min with 300 and 900 μg of protein, respectively, in the fol-

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lowing mixture: Tris–HCl buffer (pH 8.2), 50 mM; 5-phosphoribosyl-1-pyrophosphate (PRPP), 2 mM; $MgCl_2$, 2 mM; and [6-^{3}H]-5-fluorouracil (0.4 μCi), 0.5 mM; the final volume was 200 μl. After incubation of the mixture, the reaction was stopped by heating for 3 min at 100°, and the amount of [6–3H]-5-fluorouridine 5′-phosphate esters formed was measured by spotting the supernatant fraction on DEAE-discs and washing with 1 mM phosphate buffer (pH 7.0) as described previously [11].

OMP decarboxylase activity was measured in the cytosol, in the manner previously described [12]. This activity was assessed by determining the production of $^{14}CO_2$ from ^{14}COOH-labeled OMP. In final concentrations, the reaction mixture, initially 0.9 ml, contained phosphate buffer (pH 6.2), 10 mM and [7-^{14}C]OMP (0.025 μCi), 0.040 mM; the reaction was started by the addition of 0.1 ml of enzyme (cytosol). Incubations were stopped at 10, 15 and 20 min, respectively, by the addition of 1 ml of 50% trichloroacetic acid to tubes that could be quickly sealed, with filter papers included that were moistened with 0.15 ml hyamine hydroxide. The tubes were agitated in a water bath for 1 hr at 37° for complete collection of the $^{14}CO_2$ on the filter papers, which were then removed from the wells and placed in vials containing 10 ml of Ria-Solve II for assessment of radioactivity in a Searle Mark III scintillation counter.

Protein determinations were performed by the Bio-Rad assay, using bovine serum albumin as the reference protein (Bulletin No. 1051 of Bio-Rad Laboratories, Richmond, CA).

RESULTS

Activities of Urd–Cyd kinase, OMP decarboxylase and FUPRTase in human colon adenocarcinomas. A detailed kinetic study to establish the optimal conditions required for the assessment of Urd–Cyd kinase activity was reported previously [9], as were the optimal requirements for the determination of OMP decarboxylase; thus, each of the enzymes was studied under optimal conditions and with linear kinetics. Table 1 summarizes the activities of the enzymes studied only in those tumors in which the activities of the enzymes were significantly different from their mean (values within blocks and a P value less than 0.006). The mean value for Urd–Cyd kinase activity in fifty-three human colorectal adenocarcinomas was 0.86 ± 0.16 nmole uridine phosphates·min^{-1}·(mg protein)$^{-1}$. Only three specimens (Nos. 1–3) of the fifty-three exhibited activities that were significantly greater than the mean (0.86 ± 0.16). The activities of both FUPRTase and OMP decarboxylase in the three specimens (No. 1–3) shown in Table 1 were less than the mean activity of these two enzymes (represented in Table 1 by the symbol X). The mean activity of FUPRTase in thirty-five neoplasms was 0.19 ± 0.07 and only two specimens (No. 5 and No. 6) exhibited activities higher than the mean value. The activities of both Urd–Cyd kinase and OMP decarboxylase in these two specimens were less than the mean activity of each enzyme. Only one specimen (No. 4) exhibited greater OMP decarboxylase activity than that indicated by the mean value (0.21 ± 0.04); again, this specimen did not exhibit higher activities with respect to the other two enzymes studied.

Table 1 also shows that, in the population of neoplasms studied for each enzyme, a few specimens appeared to be significantly different ($P < 0.006$) from the mean of that population. In addition, it is apparent from Table 1 that the presence of a high activity of one enzyme in certain carcinomas was not accompanied by an increase in the activities of the other two enzymes.

Enzyme activities of normal human colon mucosa. The activities of the three enzymes (see above) were

Table 1. Summary of Urd–Cyd kinase, FUPRTase and OMP decarboxylase activities in those tumor specimens exhibiting apparently higher activities than the mean values*

Specimen number	Urd–Cyd kinase Mean (53)† 0.86 ± 0.16	FUPRTase Mean (35) 0.19 ± 0.07	OMP decarboxylase Mean (34) 0.21 ± 0.04
1	4.27 (P = 0.002)‡ Z = 2.9	X§	ND‖
2	4.50 (P = 0.001) Z = 3.1	X	X
3	6.4 (P < 0.001) Z = 4.7	X	X
4	X	X	1.25 (P < 0.001) Z = 4.5
5	X	1.2 (P = 0.006) Z = 2.5	X
6	X	2.05 (P < 0.001) Z = 4.5	X

* Enzymatic activities are expressed in nmoles of products·min^{-1}·(mg protein)$^{-1}$.

† Numbers within parentheses indicate the total number of specimens of colorectal adenocarcinoma analyzed for the respective enzyme activity.

‡ In a normal population, the probability of exceeding K standard deviations is easily calculated or obtained from standard tables. If the observed values are expressed in "Z-scores" (i.e. standard deviations from the mean), a Z value exceeding 2.5 has a probability of less than 0.006. Only those values within blocks were found to deviate significantly from the mean.

§ X indicates that the activity was not significantly different from the mean value.

‖ Not determined.

Table 2. Enzyme activities of human colorectal adenocarcinomas and normal colon mucosa*

	Normal colon mucosa	Colorectal adenocarcinomas	Colon tumors (% of normal colon)	P value
De novo route				
OMP decarboxylase activity				
Mean value for 34 specimens:	0.11	0.21	191	0.013†
Salvage				
Uridine–cytidine kinase activity				
Mean value for 53 specimens:	0.37	0.86	232	0.004
5-Fluorouracil phosphoribosyl-transferase activity				
Mean value for 35 specimens:	0.15	0.19	127	0.59

* Enzymatic activities are expressed in nmoles of product·min^{-1}·(mg protein)$^{-1}$.

† A two-tailed *t*-test was performed using the individual observations on the specimens of neoplastic and normal tissues. A P value that is less than 0.05 is considered to be statistically significant.

Table 3. Summary of the histopathological characteristics of the fifty-three colorectal adenocarcinomas studied

	Site distribution of tumors					Grade of differentiation			Stage of tumor*			
	Right colon	Transverse colon	Left colon	Rectum	Not specified	Well	Moderate	Poor	A	B	C	*in situ*
No. of samples	13	6	16	11	7	3	39	11	8	19	25	1

* Stage: A, tumor was confined within bowel wall; B, tumor extended into pericolonic tissue; and C, lymph node metastases were present.

also studied in samples of normal human colon mucosa. Table 2 shows that the mean value for Urd–Cyd kinase activity was 0.37 nmole of product·min^{-1}·(mg protein)$^{-1}$; for FUPRTase, 0.15; and for OMP decarboxylase, 0.11; these activities were increased by 132, 27, and 91%, respectively, as compared to the values found in normal human colonic mucosa. Also, the activities of Urd–Cyd kinase of 70% of the specimens of colorectal adenocarcinomas were higher than those of the corresponding normal tissue, while in the case of FUPRTase and OMP decarboxylase it was increased in 60 and 71% of the carcinomas, respectively (unpublished observations).

Pathologic examination of colon carcinomas. Of the fifty-three carcinomas studied, all portions of the large bowel were represented (Table 3). The specimens averaged 5.5 cm in diameter (range 2.5–10 cm), and the majority were moderately differentiated (72%). Pathologic staging disclosed 15% Dukes' A, 36% Dukes' B, 47% Dukes' C, and 2% carcinomas *in situ*. No obvious correlation was found between the level of the enzyme activities and any characteristics of the carcinoma (e.g. mucin production, sex, age, site and grade).

DISCUSSION

As a major cause of cancer death in humans, colorectal adenocarcinomas are second in frequency only to bronchiogenic carcinomas, especially in males. The failure of these neoplasms either to respond, other than temporarily, to existing chemotherapeutic agents or to be suppressed as a result of the inhibition of the biosynthesis of UMP *de novo* [13–15] may reflect the capacity of these neoplastic cells to salvage uracil, Urd and Cyd. The average activity of Urd–Cyd kinase, which is rate-limiting in the salvage of Urd and Cyd, was ~2.3 times that of normal colon mucosa ($P = 0.004$), while those of OMP decarboxylase and FUPRTase were increased by ~91% ($P = 0.01$) and by only 27% ($P = 0.59$), respectively, in those specimens of colorectal adenocarcinomas studied as compared to the values observed in the specimens of normal tissues analyzed. Only six specimens exhibited significantly higher activity, with respect to one or another of the three enzymes studied. Information regarding whether or not any of these increases in enzymic activity are related to temporary responses to chemotherapy with 5-FU (or other drugs) is not yet available. The future course of as many of these patients as possible will be followed, with special attention being given to any possible correlations between objective responses and the enzyme activities studied. The results presented here show clearly that the activity of the salvage enzyme (Urd–Cyd kinase) was higher in colorectal adenocarcinomas than in their normal counterpart (colonic mucosa), but the activity of Urd–Cyd kinase in the colorectal neoplasms was lower than in other neoplasms that have been studied in our laboratories [6–8]. The regulation and the activities of the enzymes *in vivo* may be different from those found *in vitro*, where it has been shown that Urd–Cyd kinase activity is sensitive to feedback inhibition by the end-products CTP and UTP [5]. The size of the pools of these nucleotides appears to vary during different phases of growth.

No obvious correlation between these specimens with high enzyme activities (Nos. 1–6, Table 1) and their pathological characteristics was observed. The only observation of possible pertinence was that none of the six specimens was well differentiated; five were moderately differentiated, and one was poorly differentiated.

Recent findings with neoplastic cells in culture [16] have suggested that normal plasma levels of Urd (2–12 μM) may be quite sufficient for its utilization by salvage with resultant circumvention of the *de novo* pathway to UMP [e.g. with *N*-phosphonacetyl-L-aspartic acid (PALA)]. Accordingly, the utilizations of Urd and Cyd which pass from the extracellular fluids into the intracellular milieu by facilitated diffusion [17] and are salvaged by enzymic phosphorylation (catalyzed by Urd–Cyd kinase) are of importance to chemotherapy.

The use of compounds that inhibit not only one or more steps in the *de novo* synthesis of UMP but also the salvage of Urd and Cyd offers a new approach to the chemotherapy of colorectal adenocarcinomas. Compounds of current interest that were recently described [7] include 5′-azido derivatives of both Urd and Cyd and also 5′-*O*-nitrouridine and 5′-*O*-nitro-5-fluorouridine. Some of these compounds will now be studied *in vivo*.

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