A Comparison of 5-Fluorouracil Metabolism in Human Colorectal Cancer and Colon Mucosa

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The metabolism of 5-fluorouracil (5-FU) was studied in biopsy specimens of primary colorectal cancer and healthy colonic mucosa obtained from previously untreated patients immediately after surgical removal. The conversion of 5-FU to anabolites was measured under saturating substrate (5-FU) and cosubstrate concentrations. For all enzymes, the activity was about threefold higher in tumor tissue compared with healthy mucosa of the same patient. The activity of pyrimidine nucleoside phosphorylase with deoxyribose-1-phosphate (dRib-1-P) was about tenfold higher (about 130 and 1200 nmol/hr/mg protein in tumors) than with ribose-1-phosphate (Rib-1-P), both in tumor and mucosa. Synthesis of the active nucleotides (5-fluoro-uridine-5-monophosphate [FUMP] and 5-fluoro-2-deoxyuridine-5-monophosphate [FdUMP]) was studied by adding physiologic concentrations of adenosine triphosphate (ATP) to the reaction mixture; the rate of FdUMP synthesis was 30% of that of FUMP (about 4 and 7 nmol/hr/mg protein in tumors). Direct synthesis of FUMP from 5-FU in the presence of 5-phosphoribosyl-1-pyrophosphate (PRPP) was about 2 nmol/hr/mg protein. With the natural substrate for this reaction, orotic acid, the activity was about 14-fold higher. To obtain insight into the recruitment of precursors for these cosubstrates, the authors also tested the enzyme activity of pyrimidine nucleoside phosphorylase with inosine and ribose-5-phosphate (Rib-5-P, as precursors for Rib-1-P) and deoxyinosine (as a precursor for dRib-1-P); enzyme activities were approximately 7%, 7%, and 3%, respectively, of that with the normal substrates, both in tumors and mucosa. However, when ATP and Rib-5-P were combined, the synthesis of FUMP was about 70% of that with PRPP, but only in tumors. In normal tissues no activity was detectable. These data suggest a preference of colon tumor over colon mucosa for the conversion of 5-FU to active nucleotides by a direct pathway; a selective antitumor effect of 5-FU may be related to this difference.


Fluopyrimidines are used widely for the treatment of solid tumors, such as breast, colorectal, and head and neck cancer.¹² The drug 5-fluorouracil (5-FU) has a limited effect in the treatment of advanced colorectal cancer; published response rates vary from 5% to 20%.

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Treatment is palliative, most responses are partial, and no significant effect on survival is observed. Recently, encouraging results were shown with the combination of 5-FU and leucovorin (folinic acid); the latter acts as a precursor for the folate cofactor for thymidylate synthase (TS).³ The response rates vary from 20% to 50%,⁴ with a larger number of complete responders. Several studies reported an increase in survival.³ Despite this enhanced therapeutic efficacy, many patients are refractory to 5-FU treatment. Several biochemical and biologic factors may be responsible for this resistance to fluoropyrimidines.⁵ The drug might not reach the target tissue, the tumor, or the 5-FU might not be converted to one of its active metabolites: 5-fluoro-uridine-5'-triphosphate (FUTP) or 5-
fluoro-2'deoxuryridine-5'-monophosphate (FdUMP). The metabolite FUTP can be incorporated into RNA, and FdUMP is a potent inhibitor of TS, a key enzyme in the de novo synthesis of deoxothymidine monophosphate, a precursor for DNA synthesis. We investigated the biochemical parameters that are critical in the activation of 5-FU to the active metabolites.

The activation of 5-FU to its nucleotides can proceed by the following pathways (Fig. 1): (1) directly to FUMP in a reaction catalyzed by orotate phosphoribosyltransferase (OPRT) with 5-phosphoribosyl-1-pyrophosphate (PRPP) as the cosubstrate; (2) indirectly to 5-fluorouridine-5'-monophosphate (FUMP) in a sequence of reactions with conversion of 5-FU to 5-fluorouridine (FUR) catalyzed by a pyrimidine nucleoside phosphorylase with ribose-1-phosphate (Rib-1-P) as the cosubstrate and subsequent phosphorylation to FUMP catalyzed by uridine-cytidine kinase; and (3) indirectly to FdUMP by 2'deoxy-5-fluorouridine (FdUR) with deoxy-Rib-1-P (dRib-1-P) as the cosubstrate and catalyzed by a pyrimidine nucleoside phosphorylase and thymidine kinase, respectively. For convenience, the reaction sequences discussed in (2) and (3) are referred to as the sequential conversion of 5-FU to FUMP and FdUMP, respectively; the enzymes do not exist as an enzyme complex. In all tissues 5'-nucleotidases and phosphatases may degrade the nucleotides to their inactive nucleosides. Together the activities of these enzymes and the availability of cosubstrates determine the synthesis of active 5-FU nucleotides in tumors and normal tissues. To determine whether the sensitivity or resistance of certain colon tumors is related to their enzyme pattern, we measured the activities of these enzymes in primary colon tumors from several patients not treated with chemotherapy. In addition, to obtain insight into a possible selectivity for 5-FU activation in tumor tissue compared with normal tissue, we also measured these enzymes in adjacent normal mucosa. The reliable measurement of the concentrations of cosubstrates in tissues of patients is hampered by technical problems concerning their stability and recovery during the removal of tissues. Therefore, we tested several precursors for these cosubstrates for their capacity to replace these compounds. These data suggested a preference for cancerous tissue compared with normal mucosa to convert 5-FU to the active metabolites.

Materials and Methods

Chemicals

The pyrimidines and fluoropyrimidines for biochemical purposes were purchased from Sigma (St. Louis, MO). The PRPP was obtained from Boehringer (Mannheim, Germany). Plastic sheets precoated with 0.1 mm of polyethyleneimine cellulose were obtained from Merck (Darmstadt, Germany). The 6-14C-5-FU and 14C-carboxyl-orotic acid were obtained from the Radiochemical Centre Amersham (Amersham, UK). All other chemicals were of standard analytic quality.

Enzyme Assays

Biopsy specimens of primary colorectal tumors and adjacent normal mucosa (free from underlying muscle) from untreated patients were obtained as soon as possible after surgical removal and immediately frozen and stored in liquid nitrogen. Under these conditions, the enzymes were stable for at least 3 years. Care was taken that visibly necrotic regions were not frozen. Frozen tissues were pulverized using a microdismembrator as described, allowing an excellent extraction of enzyme activities. After pulverization the powder was weighed and suspended in assay buffer (50 mmol/l tris HCl, 1 mmol/l ethylenediamine tetraacetic acid, pH 7.4) at a concentration of 1 g tissue per 3 to 4 ml of buffer. The suspension was centrifuged at 2500 x g (5 minutes, 4°C), and subsequently the supernatant was centrifuged at 11,000 x g for 10 minutes at 4°C. The supernatant was used immediately to determine enzyme activities.

All enzyme assays were done at 37°C in a water bath. Assays with 5-FU as the substrate were done as described previously for human and murine tumors. In brief, to assay pyrimidine nucleoside phosphorylases, the reaction mixture contained 2.5 mmol/l dRib-1-P, 5 mmol/l MgCl₂, and 2 to 50 μg of protein. To measure nucleotide synthesis (FUMP and FdUMP by FUR or FdUR, respectively) catalyzed by a pyrimidine nucleoside phosphorylase and subsequently a nucleoside kinase, adenosine triphosphate (ATP) was added at 2.5 mmol/l final concentration and

![Fig. 1. Schematic flow diagram of the pathways of 5-FU activation to nucleotides, including the utilization of precursors of cosubstrates. The enzymes catalyzing these reactions are as follows: 1, OPRT; 2, uridine phosphorylase; 3, thymidine phosphorylase; 4, uridine kinase; 5, thymidine kinase; 6, 5'-nucleotidases and phosphatases; 7, phosphoribomutase; 8, pyrimidine nucleoside phosphorylase; 9, PRPP synthetase; 10, ribonucleotide reductase; 11, nucleoside monophosphate and diphosphate kinases; 12, thymidylate synthase (TS); 13, RNA polymerase; and 14, DNA polymerase. TS catalyzes the formation of a ternary complex between TS, FdUMP, and 5,10-methylene-tetrahydrofolate (CH₂THF).](image-url)
more protein (50 to 250 μg) was present in the assay. To prevent breakdown of newly formed nucleotides by phosphatases, 15 mmol/l 2-glycerol-phosphate was added to these assays. For measurement of the direct conversion of 5-FU to FUMP catalyzed by OPRT, the pentose phosphates were substituted by 2 mmol/l PRPP, and 0.6 mmol/l α,β-methylene-adenosine diphosphate was added to inhibit 5'-nucleotidases; 50 to 250 μg of protein was present. All reactions were initiated by adding radiolabeled 5-FU (final concentration, 0.27 mmol/l 6-14C-5-FU with a specific activity of 4.5 mCi/mmol). The reaction time varied between 15 and 60 minutes. All assays were linear with time and protein. The reactions were stopped by heating for 3 minutes at 95°C in an Eppendorf 5320 incubator (Eppendorf, Hamburg, Germany). The products were separated from the substrate 5-FU with thin-layer chromatography. The activity of OPRT with the natural substrate orotic acid was assayed with 0.12 mmol/l 14C-carboxyl-orotic acid using the 14CO2 release method as described previously for cell lines. The assay mixture contained 50 to 2000 μg of protein, 4.5 mmol/l MgCl2, and 0.55 mmol/l PRPP. The reaction time varied between 15 and 60 minutes.

The protein content was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany) as described previously. Our results were evaluated using Student's t test for paired and unpaired data.

Results

Primary colorectal tumors and adjacent mucosa from nine patients were studied. None of the patients (median age, 56 years; range, 54 to 90) had received prior treatment with 5-FU, and the presence of colorectal cancer was proved histologically. The tumors (Dukes' classification A to C) originated from the colon transversum (three patients) and rectosigmoid (six patients). The differentiation grade varied from poor (two patients) to moderate (seven patients). Storage of the tumor samples in liquid nitrogen did not affect the enzyme activity; repeated enzyme measurements after an interval of 3 years did not show a difference in enzyme activity.

Pyrimidine nucleoside phosphorylase with Rib-1-P as the cosubstrate (uridine phosphorylase) was significantly higher in tumors than in normal mucosa from the same patients (Fig. 2). Also with dRib-1-P as the cosubstrate (thymidine phosphorylase), the enzyme activity was significantly higher in tumors than in normal mucosa. Conversion of 5-FU to either FUMP or FdUMP was measured by adding ATP at physiologic concentrations to the assay mixture for pyrimidine nucleoside phosphorylase. Under these conditions, an estimate of the conversion of 5-FU to the nucleotides can be obtained. This pathway was measured in the presence of a phosphatase inhibitor; in the assay mixture with Rib-1-P, the FUMP formation for both tumor and mucosal samples was approximately 1.6-fold higher than in the absence of the inhibitor. In the mixture with dRib-1-P, only a 1.1-fold increase was observed (data not shown). In the assay for direct formation of FUMP catalyzed by OPRT, we routinely added a nucleotidase inhibitor, resulting in a twofold increase in enzyme activity (data not shown). The addition of a phosphatase inhibitor in this assay did not increase the formation of FUMP, and the addition of a nucleotidase inhibitor to the sequential conversion reactions did not increase the synthesis of the nucleotide (data not shown). Formation of FUMP in both the sequential conversion and direct assay was approximately two to threefold higher in tumors than in normal tissues; formation of FdUMP in tumors was also two to threefold higher than in mucosa (Fig. 3). Table 1 summarizes the difference between tumors and mucosa for the various enzyme reactions.

The activity of OPRT was assayed with the natural substrate, orotic acid, and was 35.5 ± 19.5 and 16.2 ± 5.5 nmol/hr/mg protein in tumors and mucosa, respectively (means ± standard deviation for nine patients). The tumors were significantly different from normal mucosa (P < 0.02). In both tissues, the activity of OPRT with orotic acid was approximately 14-fold higher than with 5-FU.

The availability of cosubstrates may limit the conversion of 5-FU to nucleotides. However, cells and tissues may contain precursors for these cosubstrates or the precursors may be supplied externally. Ribose-5-phosphate (Rib-5-P) is a naturally occurring precursor for Rib-1-P, and inosine and deoxyinosine can be supplied externally. In addition these nucleosides may be formed during degradation of purine nucleotides. Table 2 summarizes the
FIG. 3. Activities of OPRT with 5-FU as the substrate and of the sequential conversion of 5-FU to FUMP by FUR and of 5-FU to FdUMP by FUdR. Lines connect matched pairs of tumor and normal mucosa derived from the same patient. Activities in tumors were significantly different from those in mucosa (P < 0.01 for 5-FU to FUMP via FUR; P < 0.05 for the other pathways).

The capability of these compounds to serve as precursors for the synthesis of FUR, FUdR, and FUMP. Both purine nucleosides and Rib-5-P are relatively poor precursors for the synthesis of FUR and FUdR. However, Rib-5-P, in the presence of ATP, serves as a good precursor for the synthesis of FUMP in tumors but not in mucosa. The Rib-5-P is probably an excellent precursor for the synthesis of PRPP.

Discussion

We reported a comprehensive analysis of the enzymes involved in the metabolic conversions of 5-FU leading to active metabolites. A higher rate of all enzymes was found in colon tumors compared with normal mucosa. The studies with the various precursors for the cosubstrates indicated that, of the three possible pathways for nucleotide formation, the direct conversion of 5-FU to FUMP may be the most relevant pathway in tumors. This pathway seems to be unlikely in normal mucosa. Tumors may have a preference compared with normal mucosa to convert 5-FU to active metabolites, possibly accounting for the therapeutic efficacy (although limited) of 5-FU in colon cancer along with relatively mild gastrointestinal side effects.

Measuring the enzymatic profiles and the sequential conversion reactions was a satisfactory estimate of the metabolism of 5-FU both in cell lines and tumors. In particular, the use of precursors for cosubstrates can

| TABLE 1. Activities of 5-FU Metabolizing Enzymes (With 5-Fluorouracil as the Substrate) in Normal Mucosa and Tumors |
|-------------------------------------------------|-----------------|-----------------|
| Cosubstrate(s) product | Mucosa (nmol/hr/mg protein) | Tumor (% of normal mucosa) |
| Rib-1-P | FUR | 35 ± 23 | 405 ± 252* |
| dRib-1-P | FUdR | 358 ± 161 | 334 ± 150* |
| PRPP | FUMP | 1.1 ± 0.9 | 266 ± 115 |
| Rib-1-P + ATP | FUMP | 2.4 ± 1.7 | 323 ± 172 |
| dRib-1-P + ATP | FdUMP | 1.2 ± 0.9 | 389 ± 287 |

Rib-1-P: ribose-1-phosphate; dRib-1-P: deoxyribose-1-phosphate; PRPP: 5-phosphoribosyl-1-pyrophosphate; ATP: adenosine triphosphate; FUR: 5-fluorouridine; FUdR: 2-deoxy-5-fluorouridine; FUMP: 5-fluoro-uridine-5'-monophosphate; FdUMP: 5-fluoro-2-deoxyuridine-5'-monophosphate.

Values represent means ± SD. The means of the percentages were calculated from the percentages observed in matched samples of tumors and normal mucosa of each individual patient.

* Percentages observed in Patient D (2550 for FUR and 1240 for FUdR) were not included in the calculations.
provide insight into the metabolism of 5-FU through the various pathways (Fig. 1). We concluded that phosphorylation of 5-FU toFdUMP by FUDR is not an important pathway for anabolism of 5-FU. First, the sequential conversion of 5-FU toFdUMP has a lower total capacity to catalyze these steps than the pathway toFUMP. Second, the concentration of dRib-1-P in cells and tissues is negligible compared with that of Rib-1-P.10,11 Third, an external source of dRib-1-P, i.e., deoxyninosine, does not support the synthesis of FUDR efficiently. In addition, in xenografts of human colorectal cancer, treatment with FUR increased the FdUMP levels even more than at treatment with FUDR.12 So, it may be concluded from other reports and our data that metabolism of 5-FU to FUMP, either directly or indirectly, is the major pathway of initial 5-FU anabolism in colorectal cancer. In four xenografts from human colorectal cancer, the concentrations of Rib-1-P were not significantly different and apparently high enough to support 5-FU conversion to FUR. The concentrations of PRPP in these tumors varied. Substrate concentrations and ratios between enzyme activities may determine metabolism by either the OPRT pathway or the indirect pathway. In our experiments, Rib-5-P, a direct precursor of both Rib-1-P and PRPP, appeared to be an excellent precursor for the synthesis of FUMP but not of FUR, indicating that, in tumors, Rib-5-P would be converted to PRPP rather than to Rib-1-P. This suggests that the bioavailability of PRPP in tumors might be higher than expected from steady-state concentrations. Low steady-state concentrations may reflect a high turnover rate of PRPP. No FUMP formation could be detected in normal mucosa. In murine colorectal tumors and squamous cell carcinoma of the head and neck, we found strong evidence that a preferential use of the OPRT pathway was correlated with a higher sensitivity to 5-FU treatment.5 An even better correlation was observed between OPRT activity (with 5-FU) and the response to 5′deoxy-5-fluorouridine, a compound for which we postulated that a high OPRT is essential for its conversion to active metabolites.5,6

Several authors investigated pyrimidine metabolism in colorectal cancer in humans.14-20 Usually the natural substrates for the phosphorylases and kinases were used. Because both kinetic properties (e.g., substrate specificity) and enzyme activities may be changed during neoplastic transformation,21 we measured all possible metabolic pathways of 5-FU activation with 5-FU as the substrate. It was found that the activity of uridine phosphorylase with uridine was significantly lower (60%) in tumors compared with normal tissues,18 but others observed a 2.5-fold higher activity in tumors.19 The activity of uridine phosphorylase assayed in the anabolic direction with 5-FU was fourfold higher in tumors than in normal tissue. In all studies on the activity of uridine kinase, it was two to threefold higher in tumors compared with normal mucosa.15,17-19 Also thymidine kinase, both with thymidine14,18 and FUDR20 as substrates, was two to threefold higher in tumors compared with normal tissues. A comparable activity for thymidine phosphorylase was observed with FUDR as the substrate in normal colon as we found for the conversion of 5-FU to FUDR.20 The activity of the sequential conversion of 5-FU toFdUMP agreed fairly well with the conversion of FUDR to FdUMP.20 Concentrations of FUDR in our assay exceeded the Michaelis Menten constant (Km) for phosphorylation (25 to 45 μmol/1).20 All authors observed a significantly higher activity of OPRT in tumors compared with normal mucosa, both with the natural substrate orotic acid (two to tenfold) and 5-FU (approximately twofold higher).14,17-19 The activities of uridine and thymidine phosphorylase with 5-FU as a substrate and the sequential reactions were all two to three times higher in gastric carcinoma22,23 compared with normal tissues. In addition higher activity was found in poorly differentiated tumors compared with well-differentiated tumors. Unfortunately we did not obtain the latter.

Most authors assayed the enzymes in clarified supernatants, and their activity was expressed as nmol of product formed per hour (or minute) per mg protein (or per ng DNA). The relative pattern of enzyme activities usually agreed with the one we found, but large differences in absolute values were observed. All enzyme activities (uridine phosphorylase and uridine and thymidine kinase and OPRT) reported by some14,19 were at least ten to 1000-

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Normal cosubstrate</th>
<th>Product</th>
<th>Tumor</th>
<th>Mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inosine</td>
<td>Rib-1-P</td>
<td>FUR*</td>
<td>7.8 ± 4.4 (8)</td>
<td>7.1 ± 5.3 (7)</td>
</tr>
<tr>
<td>Deoxyinosine</td>
<td>dRib-1-P</td>
<td>FUr†</td>
<td>2.6 ± 2.1 (7)</td>
<td>1.6 ± 0.8 (8)</td>
</tr>
<tr>
<td>Rib-5-P</td>
<td>Rib-1-P</td>
<td>7.3 ± 5.2 (9)</td>
<td>7.5 ± 4.2 (6)</td>
<td></td>
</tr>
<tr>
<td>Rib-5-P + ATP</td>
<td>PRPP</td>
<td>FUMP†</td>
<td>71.9 ± 26 (3)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Rib-5-P: ribose-5-phosphate; ATP: adenosine triphosphate; Rib-1-P: ribose-1-phosphate; dRib-1-P: deoxyribose-1-pyrophosphate; PRPP: 5-phosphoribosyl-1-pyrophosphate; FUR: 5-fluorouridine; FdUra: 2′deoxy-5-fluorouridine; FUMP: 5-fluoro-uridine-5′-monophosphate.

Absolute activities in tumors were significantly different from that in mucosa at the level: *P < 0.001; †P < 0.02; ‡P < 0.05.
fold higher than those reported by us and others.\textsuperscript{13,15-18,20}
This is probably related to the fact that different enzyme preparations, substrates, and assays were used. In xenografts of human colorectal cancer,\textsuperscript{13,17} the range of the activities of uridine kinase, uridine phosphorylase, and OPRT were in the same range as the activities we and others\textsuperscript{15-18} observed in samples obtained from patients.

For a complete analysis of 5-FU metabolism in human tumors, sensitive and selective methods (usually using radiolabeled compounds) are a prerequisite. In one report, 5-FU metabolism was studied in colorectal tumors after treatment with radiolabeled 5FU; the formation of FUR and nucleotides was found as was their incorporation into RNA.\textsuperscript{24} Analysis of tissue levels of 5-FU and some metabolites was done with high-performance liquid chromatography,\textsuperscript{25} \textsuperscript{19}F compounds in nuclear magnetic resonance,\textsuperscript{26} and gas chromatography coupled to mass spectrometry.\textsuperscript{27} Long retention of 5-FU was shown after a bolus injection of 5-FU.\textsuperscript{24} Preliminary data from our laboratory\textsuperscript{27} demonstrated that 5-FU is present in both tumors and normal tissues (livers and colon mucosa) at concentrations varying from 5 to 20 \textmu mol/l, even 24 hours after a bolus injection of 5-FU at a dose of 500 mg/m\textsuperscript{2}.

Because of the preferential metabolism of 5-FU to active metabolites, it is unlikely that the variation in response is related to its anabolism in tumors. Other factors, such as the activity and kinetic properties of TS\textsuperscript{2,5} may be responsible. In contrast to most other studies, we were able to measure the activity of this enzyme\textsuperscript{28} in the same tumors. We observed a large variation, both in catalytic activity and inhibition byFdUMP. A variation based on histologic heterogeneity of samples is unlikely because the same homogenate was used. A biochemical homogeneity for several pyrimidine enzymes seems to be present, excluding TS.\textsuperscript{29,30} The administration of leucovorin may increase the extent of inhibition of this enzyme in patients with a high TS.

The preferential use of the direct pathway of 5-FU metabolism to FUMP may be used to improve the therapeutic efficacy of 5-FU. Encouraging data recently were reported by O'Dwyer et al.\textsuperscript{31} who showed a higher response rate for the combination of 5-FU with phosphonacetyl-L-aspartate, a compound that may increase PRPP availability,\textsuperscript{9} leading to enhanced metabolism of 5-FU. Combinations of such approaches with those aiming at modulation of TS may lead to an additional or synergistic effect.

REFERENCES

23. Maehara Y, Kusumoto T, Emi Y et al. 5-Fluorouracil is converted to F-nucleotides more extensively and is more cytotoxic in poorly differentiated than in well differentiated human gastric carcinoma. *Anti-cancer Res* 1990; 10:1091–1094.


