Pyrimidine nucleoside phosphorylase activity in tumour and matched normal gastrointestinal mucosa

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SUMMARY 5-Fluorouracil (5-FU) requires activation to the metabolites FdUMP and FUTP for its cytotoxic effect. This activation involves the intracellular enzymes uridine and thymidine phosphorylase. We have assayed the levels of these enzymes in colorectal and gastric cancers and have shown that, in the majority of cases, the enzyme levels were higher than in the adjacent normal mucosa. It is suggested that, while the ratio tumour/normal mucosa enzyme activity may give some indication of the relative toxicity of 5-FU in these tissues, the absolute activities of the phosphorylases in tumour tissue could give a better indication of tumour responsiveness.

5-Fluorouracil (5-FU) remains the most effective of the currently available cytotoxic drugs for use against gastrointestinal tumours.¹⁻³ Only 20% of patients with advanced cancer, however, show a definitive response which, although short-lived, does double the mean survival time of responders over non-responders or untreated patients. As such a small percentage of patients respond to 5-FU therapy it is clearly desirable to identify those patients who are likely to respond and to avoid unnecessary treatment in the remainder.

It is generally accepted that 5-FU itself has no direct cytotoxic activity but exerts its influence *via* its active metabolites 5-fluorodeoxyuridine-5'-monophosphate (FdUMP) which inhibits thymidylate synthetase activity thereby starving cells of TMP for DNA synthesis⁴ and 5-fluorouridine-5'-triphosphate (FUTP) which becomes incorporated into RNA and seems to affect both RNA maturation and DNA synthesis. Recent evidence would seem to suggest that possibly it is the second metabolite FUTP which may exert the major cytotoxic influence.^{5,6} As nucleotides do not readily cross cell membranes it is logical to assume that activation will occur intracellularly and that the effect of the metabolites will be restricted to the activating cell.

Much work has been published attempting to relate cellular sensitivity to 5-FU with the activity of enzymes thought to be involved in this activation process.⁷⁻¹⁰ The enzymes uridine phosphorylase, uridine kinase, uracil phosphoribosyl transferase, and the nucleotide kinases are common for the activation of 5-FU to both FdUMP and FUTP

(Figure). Thymidine phosphorylase and kinase and ribonucleotide reductase are more exclusively involved with activation to FdUMP. In this study we have assayed the levels of two of these activating enzymes uridine and thymidine phosphorylase within gastrointestinal mucosa and within matched samples of gastrointestinal carcinomas to determine whether the enzymes display a markedly different activity in the two tissues.

Methods

MATERIALS

Nucleosides were obtained from Sigma Chemical Co. Ltd. and 5-FU sodium salt from Hoffman La Roche. HPLC grade solvents from BDH or Fisons were used where indicated. All other reagents were of analytical grade obtained from BDH.

PATIENTS AND PATIENT SAMPLING

Twenty eight patients were included in the

Fig. Possible routes of 5-FU activation. Key to enzymes: (1) thymidine phosphorylase; (2) uridine phosphorylase; (3) uracil phosphoribosyltransferase; (4) uridine kinase; (5) thymidine kinase; (6), (7) nucleoside monophosphate kinase; (8) ribonucleotide reductase; (9) nucleoside diphosphate kinase.

experiments, 15 males and 13 females. All were patients with gastrointestinal tumours and were subjected to surgical excision of the primary tumour. Fifteen of the patients received 5-FU from one to four days before operation and at this time most (13 patients) had the plasma pharmacokinetics of the drug monitored. The remaining 13 patients received no 5-FU. Tumour site was divided as follows: gastric, three; right colon, five; colon, seven; rectum, 13.

After the specimen was removed a piece of tumour (0.5 g minimum) and of normal mucosa (if possible no closer than 10 cm to the tumour) were randomly cut from it as soon as possible, frozen in liquid nitrogen, and stored at -70°C for subsequent analysis.

The mean age of the patient group of 15 males and 13 females was 64.5 years (range 42–82 years). After pathological examination of the resected specimen, all three gastric cancers proved to have lymph node metastases and the patients with colorectal cancer were staged as Dukes's B (12 patients); Dukes's C (10 patients). The three remaining patients had liver metastases.

ENZYMATIC ANALYSIS

A crude homogenate of tissue was prepared by weighing approximately 0.1 g tissue and homogenising it in 4 volumes of homogenising medium (50 mM potassium phosphate + 1 mM EDTA + 5 mM β -mercaptoethanol pH 7.0). The homogenate was centrifuged at $100,000\times g$ for 60 minutes at 4°C and the supernatant was then dialysed overnight against 500 volumes of homogenising buffer. The dialysed supernatant was used in the enzymatic reaction. The incubation medium contained at final concentration 75 mM phosphate buffer (pH 7.5), 5 mM substrate (thymidine, deoxyuridine or uridine), and tissue extract in a final volume of 300 μl . The reaction, performed at 37°C , was started by the addition of 50 μl tissue extract and stopped at 0, 10, 20, and 30 minutes by the removal of 50 μl ; this was then added to 50 μl ice cold 0.8M perchloric acid. After protein precipitation and removal, 50 μl of the resultant supernatant was added to 50 μl of 0.8 mM sodium bisulphite. This was stored at -70°C for subsequent HPLC analysis for uracil or thymine as appropriate. The protein content of the tissue extract was determined by the biuret method. Activity is expressed as nmol base produced/mg protein/h.

HPLC DETERMINATION OF URACIL OR THYMINE PRODUCTION

Analyses were performed using a system consisting of a ACS 750/03 pump with a fixed wavelength UV

detector 750/11 set at 254 nm connected to a Houston omniscrite chart recorder. Loading was manual using a Rheodyne 7125 injector with 10 μl loop (Applied Chromatography Supplies, Luton, Beds.). Data reduction was by manual peak height measurement. More recently the analysis has used a LDC constrametric III pump, spectromonitor III variable UV detector set at 254 nm, loading was with a Magnus Autosampler (Magnus Scientific) fitted with a 20 μl loop and data reduction was by automated peak height measurement using a CCM (Lab. Data Control (UK), Stone, Staffs.). Samples were eluted from a 250 $\times$ 4 mm Spherisorb S5 ODS column using either phosphoric acid pH 3.0 at 1.0 ml/min in the case of deoxyuridine and uridine phosphorylase, or 50 mM phosphate buffer pH 3.0 containing 10% methanol in the case of thymidine phosphorylase. Although elution volumes on the two systems (ACS vs LDC) varied a little, results were reproducible within 1% provided that the standards, run through each appropriate system, were used for quantification. External standardisation using uracil or thymine standards run daily were used throughout.

STATISTICAL ANALYSIS

Data were analysed by a modified rank-sum test (unpaired) or Wilcoxon signed rank test (paired) where appropriate. the degree of correlation was ascertained by Spearman's rank correlation coefficient. Significance was accepted at $p<0.06$.

Results

EFFECT OF AGE ON PHOSPHORYLASE LEVELS

There was no significant correlation between the age of the patient and the tissue levels of deoxyuridine phosphorylase or uridine phosphorylase in normal or tumour tissue nor when the tumour/normal mucosal ratio was calculated. There was, however, a degree of positive correlation between increasing age and thymidine phosphorylase in normal mucosa ($R_s=0.478$, $p<0.02$) but not tumour tissue.

EFFECT OF SEX OF PATIENT

There was no statistical difference between the results obtained for males and females with regard to any of the phosphorylase activities measured in either tumour or normal mucosa, although females did display a slightly higher average activity than males in both tissues and with all three phosphorylases. The range of phosphorylase levels in normal and cancerous tissue is shown in Table 1.

EFFECT OF MUCOSAL SITE

In normal mucosa deoxyuridine phosphorylase was

Table 1 *Pyrimidine nucleoside phosphorylase activity*

	Uridine phosphorylase		Thymidine phosphorylase		Deoxyuridine phosphorylase	
	Tumour	Normal mucosa	Tumour	Normal mucosa	Tumour	Normal mucosa
All patients (28)	90.0±48.8*	63.7±41.8	397.6±208.2	292.1±139.3	792.2±285.7	439.1±218.9
Post-5-FU (15)	89.0±50.1	61.7±35.1	404.3±209.2	276.2±131.8	695.0±367.9	347.6±152.8
No 5-FU (13)	91.1±49.3	65.9±49.8	389.8±215.3	310.5±150.7	904.3±389.1	544.7±240.9
Tumour site						
Stomach (3)	77.3±64.3	19.0±12.5	222.3±65.0	190.6±108.5	644.2±442.2	279.5±170.6
Caecum (5)	76.7±29.5	83.4±70.7	374.9±178.3	384.2±163.5	780.8±260.9	637.4±150.8
Colon (7)	122.2±62.3	71.6±15.6	474.3±292.6	277.9±105.9	932.3±624.7	427.0±225.0
Rectum (13)	80.6±40.3	62.1±37.6	405.4±179.0	287.8±144.7	755.2±256.7	406.2±214.6

* Mean ± standard deviation.

significantly higher ($p<0.05$) in the caecum than in either stomach or rectum (637.4 ± 150.8 ; 279.5 ± 170.6 and 406.2 ± 214.6 respectively) and also higher than the rest of the colon ($p<0.053$). Uridine phosphorylase was significantly lower ($p<0.001$) in the stomach than colon (71.6 ± 15.6 and 19.0 ± 12.5 respectively) and thymidine phosphorylase activity was only significantly higher in caecum than either stomach or rectum ($p<0.036$ and $p<0.028$ respectively) and lower in rectum than colon ($p<0.026$). It was found that the only statistically significant difference between tumours from different sites of the gut was that thymidine phosphorylase activity was lower in gastric than in colorectal cancer (<0.058).

EFFECT OF TUMOUR STAGE

With respect to Dukes's classification, deoxyuridine phosphorylase activity was greater in tumour tissue of Dukes's B patients than Dukes's D patients (831.6 ± 266.3 ; 210.8 ± 180.4 respectively, $p<0.05$) and all three enzyme activities were lower in the tumours of Dukes's D patients than in Dukes's C patients (thymidine, $p<0.056$; deoxyuridine, $p<0.056$; uridine, $p<0.032$). There were no differences between Dukes's classification patients for any of the phosphorylase activities in normal mucosa.

EFFECT OF 5-FU THERAPY

It was noted that the average activity of the enzymes was slightly lower in patients who had received 5-fluorouracil before surgery than those who had not, but that this difference was statistically significant ($p<0.05$) only for deoxyuridine phosphorylase activity in normal mucosa.

COMPARISON OF TUMOUR TISSUE WITH NORMAL MUCOSA

In the majority of cases phosphorylase activity was

higher (ratio >1.1) in tumour than in normal mucosa (thymidine, 16/28; deoxyuridine, 21/23; uridine, 19/28). This was true both for males (8/15, 11/14, and 9/15) and females (8/13, 10/13, and 10/13). An attempt was made to discern whether there was any correlation between pyrimidine nucleoside phosphorylase activity in the tumour and normal mucosa and it was found that there was some correlation ($R_s=0.561$, $p<0.02$) with respect to thymidine phosphorylase but not deoxyuridine or uridine phosphorylase.

It would seem that results obtained for the three enzymes differ somewhat qualitatively as well as quantitatively and for this reason Spearman's rank correlation was used to compare the activities of the different phosphorylases in the different tissues and the ratios obtained. It was found that there was a good degree of correlation between the activities of all three enzymes in tumour tissue (thymidine vs deoxyuridine, $R_s=0.762$; deoxyuridine vs uridine, $R_s=0.626$; uridine vs thymidine, $R_s=0.579$; for all $p<0.02$) but that in normal mucosa there was only good correlation between thymidine and deoxyuridine ($R_s=0.739$, $p<0.02$) and very poor correlation between thymidine and uridine ($R_s=0.337$, $p<0.1$) and between uridine and deoxyuridine ($R_s=0.373$, $p<0.1$).

This pattern of correlation in the normal mucosa was mirrored by the tumour/normal mucosa ratio (Table 2) in which there was good correlation between thymidine and deoxyuridine ($R_s=0.787$, $p<0.02$) but poorer correlation between thymidine and uridine ($R_s=0.354$, $p<0.1$) and between uridine and deoxyuridine ($R_s=0.379$, $p<0.1$). The above would seem to indicate that possibly the ratio is more dependent on changes in the normal mucosa activity of these enzymes than on their activity in tumour tissue and, in fact, when rank correlations were performed of tumour vs ratio and normal mucosa vs ratio it was found that the correlation was

Table 2 Ratio of pyrimidine nucleoside phosphorylase activities in tumour vs paired normal mucosa

Tumour/ normal mucosa	No. patients					
	Thymidine phosphorylase		Deoxyuridine phosphorylase		Uridine phosphorylase	
	No 5-FU	5-FU	No 5-FU	5-FU	No 5-FU	5-FU
0-5	0	0	0	0	2	0
0.51-0.9	2	5	1	3	1	4
0.91-1.09	3	1	3	0	1	1
1.10-1.5	3	3	1	2	2	1
1.51-2.0	1	0	1	3	1	4
2.01-2.5	2	3	3	1	3	2
2.51-3.0	0	0	2	2	0	1
>3.0	1	3	2	4	3	2

always better with normal mucosa than with tumour (thymidine-tumour, $R_s=0.400$; normal mucosa, $R_s=0.436$; deoxyuridine-tumour, $R_s=0.478$; normal mucosa, $R_s=0.677$; uridine-tumour, $R_s=0.296$; normal mucosa, $R_s=0.709$).

Discussion

At this stage there is not enough clinical follow-up information on the 28 patients in this study to allow for any conclusions to be reached about the possible usefulness of pyrimidine nucleoside phosphorylase activity as a predictor of tumour response to 5-FU. Nor has the study revealed a clearly defined subgroup of patients with greatly increased or decreased activity of any of the enzymes measured in either tissue or in the tumour/normal mucosa ratio which could be postulated to correspond with responder or non-responder status. It would seem likely, in view of the fact that equilibrium for these enzymes favour nucleoside formation, that a higher enzyme activity in the presence of sufficient pentose-1-phosphate could correlate with enhanced tumour sensitivity.¹⁰

It is interesting to note that tumour site does not seem to affect the activity of the pyrimidine nucleoside phosphorylases. The fact that tumour levels of the enzymes are higher than in normal mucosa may be an indication that the tumours are growing faster or capable of more rapid replication than the surrounding mucosa. Thymidine phosphorylase tissue distribution studies¹¹ would seem to indicate that an increased relative growth rate is associated with increased activity of the phosphorylase but it is not clear whether this is due to an increase in the 'salvage' function or to the catabolic activity acting as a damper on the increase in thymidine concentration (thymidine is known to

inhibit cell growth at high concentrations).

A number of studies have demonstrated that tissue from a variety of sources contains more than one pyrimidine nucleoside phosphorylase¹²⁻¹⁴ and that the spectrum of activity with the different substrates (uridine, deoxyuridine, and thymidine) varies with the differing enzyme or isoenzyme. The present study would seem to indicate that there is possibly more than one enzyme involved in the present system. Yamada¹² has suggested that thymidine phosphorylase from rat liver will display activity with both thymidine and deoxyuridine, while the uridine phosphorylases can use all three substrates. If this is so in the human intestine, then it would seem from the correlation data presented here that thymidine phosphorylase and uridine phosphorylase are making a different contribution to total pyrimidine nucleoside phosphorylase activity in tumour and normal mucosa. In the present study it appears that the predominant enzyme in tumour tissue is probably uridine phosphorylase because there was enhanced activity against all three substrates. In normal mucosa, however, the major contribution would seem to be from thymidine phosphorylase, as there was only good correlation between the activities with thymidine and deoxyuridine as substrate.

When the activity of pyrimidine nucleoside phosphorylase in tumour and in normal mucosa is correlated with the ratio of activities in the two tissues we found that the negative correlation with normal mucosa was always slightly better than the positive correlation with tumour activity. This was particularly so when the substrate was uridine or deoxyuridine. From this it might be concluded that in the case of pyrimidine nucleoside phosphorylase the tumour/normal mucosa ratio is more dependent on differences in the activity of the enzyme in normal mucosa than in tumour and hence that, while the ratio may be a useful indicator of the relative potential toxicity of 5-FU to tumour and normal mucosa, the absolute tumour activity might be a better indicator of tumour responsiveness.

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