5-Fluorouracil Metabolite Patterns in Viable and Necrotic Tumor Areas of Murine Colon Carcinoma Determined by $^{19}$F NMR Spectroscopy

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High-resolution $^{19}$F NMR spectroscopy at 9.4 T was used to study the difference in the metabolite pattern of 5-fluorouracil (5-FU) between viable and necrotic tissues of C38 murine colon tumors grown in C57Bl/6 mice. Studies were performed on perchloric acid extracts of these tumor fractions after 5-FU treatment. The $^{19}$F nuclear magnetic resonance spectra exhibited resonances representing 5-FU, the catabolites $\alpha$-fluoro-$\beta$-ureidopropionic acid and $\alpha$-fluoro-$\beta$-alanine, as well as several fluoronucleotide anabolites. The absolute concentrations of anabolites and catabolites and the anabolite-catabolite ratio were significantly lower in the necrotic fraction than in the viable tumor fraction 50 min after administration of 5-FU, whereas the absolute concentration of 5-FU was the same. Therefore, in 5-FU metabolism studies with NMR spectroscopy, it is important to consider the necrotic contribution to the tumor volume.

Key words: $^{19}$F NMR spectroscopy; 5-fluouracil; necrosis; metabolism.

INTRODUCTION

5-Fluorouracil (5-FU) is still the most important cytostatic drug in the treatment of colorectal cancer (1). To perform its cytostatic effect, it has to be converted into active anabolites (2). Furthermore, 5-FU is degraded to inactive catabolites. The first conversion occurs predominantly in tumor cells, the second mostly in the liver (3). Only a small percentage of the 5-FU is taken up in tumor cells and converted to the therapeutically important anabolites: the fluoronucleosides (FNucTs) and fluoronucleotides (FNucTs). The functionally most important FNucTs are 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP) and 5-fluorouridine 5'-triphosphate, the latter generally occurring at a much higher concentration (4). A large fraction of the administered 5-FU is degraded to catabolites of which 5,6-dihydro-5-fluorouracil (DHFU), $\alpha$-fluoro-$\beta$-ureidopropionic acid (FUPA), and $\alpha$-fluoro-$\beta$-alanine (FBAL) are the most important. Although it is generally agreed upon that the FNucTs formed as a result of anabolism are responsible for the therapeutic effect of 5-FU, the precise mechanism underlying the antitumor activity of the FNucTs is still a matter of debate (1, 2).

To understand the effectiveness of 5-FU, its metabolism has been studied in several tumor models and in patients. $^{19}$F NMR spectroscopy is one of the methods to monitor the presence of 5-FU and a number of its anabolites and catabolites in tumor tissue (4). We have recently used this method to demonstrate the effect of various biomodulators on 5-FU metabolism in murine colon carcinomas (5). In vivo NMR studies have actually correlated FNucT levels with therapeutic response (6-8). Commonly, in these studies, whole tumors are investigated and the amount of necrosis is neglected, although tumors may contain various quantities of necrosis that could affect the results.

The objective of the present study was to investigate to what extent the amount of necrosis affects $^{19}$F NMR spectral profiles. The level of 5-FU and its metabolites may be different in the necrotic part, compared with the viable part of the tumor. To address this problem, viable and necrotic parts of a murine colon tumor were separated after administration of 5-FU and $^{19}$F NMR spectra of extracts of this material were measured.

MATERIALS AND METHODS

Tumor Model

Female C57Bl/6 mice of 8 to 12 weeks of age were obtained from the Central Animal Laboratory of our university. The C38 murine colon tumor, which is known to be sensitive to 5-FU, was acquired from Dr. P. Leliweveld of the REPOG-TNO Institute (Rijswijk, The Netherlands) and is described elsewhere (9). Tumor tissue fragments with a diameter of 3 mm were implanted subcutaneously in the right flank of the mouse. To investigate the influence of tumor size and thereby amount of necrosis, three groups of tumors of different weight were measured. In the first group ($n = 6$), the experiment was performed after 20 days, when the tumors reached a weight of 1.9 (mean) ± 0.2 g (SE) necrosis included; in the second group ($n = 8$) after 27 days, with a total tumor weight of...
4.6 ± 0.6 g; and in the third group (n = 2) after 34 days, with a total tumor weight of 7.0 ± 0.05 g. No functional compromise of the mouse by the tumor burden was observed. In addition, to get insight in the pharmacokinetics of 5-FU, a time curve with respect to time after 5-FU administration was determined for 5-FU, anabolites and catabolites for the first weight group with the smallest tumors. The tumor was considered to be an ellipsoid, and its volume was estimated from three orthogonal diameter measurements (tumor volume in cubic millimeters = X × Y × Z × 0.5) (9). For the time curve, mice were divided into groups based on estimated tumor volume, and stratified randomization was conducted over the different time groups.

Chemotherapy

At the time of the experiment, the tumor-bearing mice received 5-FU at a dose of 115 mg/kg as bolus ip within 5 s. 5-FU was purchased from Roche (Mijdrecht, The Netherlands). The dose of 5-FU used (i.e., 115 mg/kg) is below the maximal tolerable dose (150 mg/kg) (10) and corresponds to 350 mg/m² in man (11), which lies in the therapeutic range (370–500 mg/m² for 5 days) (12).

Animals were killed by cervical dislocation 50 min after administration of 5-FU, except for the time curve study, because the various concentrations were measured at different time points. This time point was chosen because of active and considerable metabolite formation, as followed from a time curve of whole tumors in our previous study (5). The tumor was excised within 2 min and cleaved on ice to separate the necrotic tissue from the viable tumor tissue. Viable tumor and necrotic tissues were immediately frozen in liquid nitrogen until further processed by perchloric acid (PCA) extraction.

PCA Extraction

The frozen tissue was pulverized in a liquid nitrogen-chilled stainless steel mortar and ground to a fine powder. Per gram of pulverized tissue, 5 ml of cold 0.9 M of PCA was added, and the sample was stirred with a glass rod and thoroughly mixed (1,500 rpm, 20 strokes). After homogenization, the sample was incubated for at least 1 h in a cold chamber on a shaker. The sample was centrifuged, the pellet was discarded, and the supernatant was neutralized with KOH and 4% (v/v) of a potassium phosphate buffer (pH 6.5, 1 M, final concentration 40 mM) before freeze-drying. After freeze-drying, the lyophilisate was dissolved in 0.8 ml of demineralized water, and the pH of the sample was adjusted to 6.5, and the sample was freeze-dried again.

19F NMR Spectroscopy

19F NMR spectra were measured on a Bruker AMX 400 using a dedicated 19F probe. Freeze-dried sample residues were dissolved in 0.4 ml of demineralized water and placed in a 5-mm NMR tube, together with a coaxial insert containing 2H2O as lock substance, as well as parafluorobenzoic acid as an internal standard. Between 3,000 and 30,000 transients were accumulated, depending on the signal-to-noise ratio required and the concentration of the fluorine-containing compounds in the sample. Data acquisition parameters were: sweep width, 60 kHz; flip angle, 45° with a pulse width of 6 μs; repetition time, 1.5 s; and temperature 298°K. No 1H decoupling was used.

Analysis of 19F NMR Spectra

The 19F NMR resonance frequency of 5-FU was set to a chemical shift value of 0 ppm. The integrals of the signals of 5-FU (F), the FU anabolites (A) (FNuc + plus any FNuc), and the FU catabolites (C) (FUPA and FBAL) were calculated by comparison of the integrals of their signals to the integral of the 19F NMR resonance of the internal standard parafluorobenzoic acid (59.02 ppm). Under the current conditions, no important saturation of the resonances of A, C, and F was observed. This is concluded from an experiment in which the repetition time was set to 9 s instead of 1.5 s, but in which all other parameters were left unchanged. Relative comparison of the two spectra showed that the highest increase in signal intensity was observed for the internal standard when the repetition time was changed from 1.5 to 9 s. The internal standard was calibrated by adding a known concentration of 5-FU to an untreated tumor extract holding the reference capillary. The detection threshold of fluorinated compounds is estimated to be ~3 μM in the extract solution. Peak areas were obtained by fitting a Lorentzian model function using NMRl software (New Methods Research, Inc., Syracuse, NY).

Statistics

For each group, mean values of concentrations have been presented with their standard error of the mean (SE). Differences in mean values between viable and necrotic tumor fractions were analyzed by the paired t-test (two-sided, α = 0.05) after log transformation (13).

RESULTS

19F NMR Detection of 5-FU Metabolite Patterns in Viable and Necrotic Tumor Tissues

Figure 1a presents the 19F NMR spectrum of the extract of viable tumor tissue of a mouse 50 min after 5-FU treatment. In addition to the peak of 5-FU (Peak 2 at 0 ppm), anabolite and catabolite peaks are seen. Various FNuc peaks indicated with 1 are observed between 3 and 5 ppm. In most spectra, three, sometimes four, different peaks can be distinguished, namely at 4.57 ± 0.03, 4.31 ± 0.03, 3.77 ± 0.00, and 3.74 ± 0.06 (not visible in Fig. 1) ppm (±mean deviation). More detailed identification of the various nucleotide peaks might be possible on the basis of comparison with literature data (14–16) and with our own identification of FDUMP [small right peak from Group 1] and of the nucleoside peaks 5-fluorouridine and 5-fluoro-2'-deoxyuridine (upfield from Group 1; not visible in Fig. 1) (5). However, chemical shifts reported seem to vary from one publication to another, because of the variation in sample conditions. Specific identification of the other anabolite peaks requires the synthesis of appropriate reference compounds. For anabolites, the
area under the curve of the total FNuct peak at ~4 ppm is taken. At $-17.01 \pm 0.11$ and $-18.61 \pm 0.10$ ppm, upfield from 5-FU, two catabolite peaks are seen: FUPA (Peak 3) and FBAL (Peak 4), respectively. Sometimes two other small catabolite peaks are visible at $-33$ ppm (DHFU) and at $49$ ppm (F-cations), not shown in this spectrum. The main amount of catabolites, however, is composed of FUPA and FBAL, which in this study are referred to the catabolites.

Figure 1b presents the spectrum of the extract from the excised necrotic area belonging to the same tumor. The spectrum shows signals for the major compounds also observed in the spectrum of the viable tumor tissue extract. However, relative and absolute signal intensities are clearly different.

**Parameters for Response to Treatment**

Possible parameters that may reflect the effectiveness of 5-FU treatment are the absolute concentration of anabolites (A) representing cytotoxicity (4), of catabolites (C) representing detoxification, of total fluorine-containing compounds (T) including A, C, and F (14), the ratio of anabolites to 5-FU (A/F) (6), of anabolites to catabolites (A/C), and of anabolites to total fluorine-containing compounds (A/T) (17). In the present study, these parameters were evaluated both in viable and necrotic tumor tissue.

Table 1 summarizes the evaluation of all data at 50 min after 5-FU injection. From the table, it follows that in viable tumor tissue the absolute concentration of A is significantly higher than in the necrotic area. For the three weight groups together, these values are $88 \pm 10$, compared with $26 \pm 5$ nmol/g, $P < 0.01$. This is also the case for the absolute concentration of C ($104 \pm 8$, compared with $64 \pm 8$ nmol/g, $P < 0.05$), but not for the absolute concentration of F that seems similar in tumor and necrosis ($75 \pm 15$, compared with $68 \pm 11$ nmol/g).

The total concentration of fluorine-containing compounds T is higher in the tumor fraction, compared with necrosis ($266 \pm 22$, compared with $157 \pm 18$ nmol/g, $P < 0.01$).

Because of the different relative changes between tumor and necrosis of metabolite concentrations, some of the metabolite ratio's are also different. Higher values are found in tumor tissue for the A/C ratio ($0.91 \pm 0.13$, compared with $0.48 \pm 0.09$, $P < 0.01$) and the A/T ratio ($0.32 \pm 0.03$, compared with $0.14 \pm 0.02$, $P < 0.01$). The
Table 1

Concentration Anabolites (A), Catabolites (C), 5-FU (F), and Total Fluorine-Containing Compounds (T) and Ratios A/T, C/T, F/T, and A/C in Extracts of Viable Tumor Tissue and Necrosis of C38 Murine Colon Tumor Excised 50 Min After 5-FU Injection

<table>
<thead>
<tr>
<th>Tumor tissue</th>
<th>Day^a</th>
<th>Weight^b</th>
<th>No. of animals</th>
<th>Metabolite concentrations in nmol/g^c tissue ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Viable</td>
<td>20</td>
<td>1.3 ± 0.2</td>
<td>6</td>
<td>107 ± 14^d</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 ± 0.1</td>
<td></td>
<td>23 ± 8</td>
</tr>
<tr>
<td>Necrotic</td>
<td>27</td>
<td>3.3 ± 0.4</td>
<td>8</td>
<td>81 ± 14^d</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.4 ± 0.3</td>
<td></td>
<td>24 ± 8</td>
</tr>
<tr>
<td>All tumors together^d</td>
<td></td>
<td></td>
<td></td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>Viable</td>
<td></td>
<td></td>
<td>16</td>
<td>88 ± 10^d</td>
</tr>
<tr>
<td>Necrotic</td>
<td></td>
<td></td>
<td></td>
<td>23 ± 10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Weight</th>
<th>A/T</th>
<th>C/T</th>
<th>F/T</th>
<th>A/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable</td>
<td>1.3</td>
<td>0.40 ± 0.02^a</td>
<td>0.41 ± 0.06</td>
<td>0.19 ± 0.05^d</td>
</tr>
<tr>
<td>Necrotic</td>
<td>0.5</td>
<td>0.15 ± 0.05</td>
<td>0.46 ± 0.07</td>
<td>0.39 ± 0.05</td>
</tr>
<tr>
<td>Viable</td>
<td>3.3</td>
<td>0.28 ± 0.05</td>
<td>0.43 ± 0.08</td>
<td>0.28 ± 0.06</td>
</tr>
<tr>
<td>Necrotic</td>
<td>1.4</td>
<td>0.14 ± 0.03</td>
<td>0.41 ± 0.10</td>
<td>0.46 ± 0.09</td>
</tr>
<tr>
<td>All tumors together</td>
<td>2.7</td>
<td>0.32 ± 0.03^d</td>
<td>0.43 ± 0.05</td>
<td>0.25 ± 0.04^d</td>
</tr>
<tr>
<td>Viable</td>
<td>1.2</td>
<td>0.14 ± 0.02</td>
<td>0.44 ± 0.05</td>
<td>0.42 ± 0.05</td>
</tr>
</tbody>
</table>

^a Days after tumor transplantation.
^b Weight of viable tumor tissue and necrosis separately.
^c Quantities are given in nmol/g of extracted tissue. To achieve absolute amounts in nmoles, integrals have been multiplied by the conversion factor 104.8 (282 μM x 0.4 ml).
^d Significantly different, P ≤ 0.01.
^e Significantly different, P ≤ 0.05.
^f Including the third group (34 days, n = 2) not shown separately.

F/T ratio is even significantly lower in the tumor fraction (0.25 ± 0.04, compared with 0.42 ± 0.05, P < 0.01). The same tendency is seen in the separate weight groups.

Histological Determination of Percentage Viable Tumor Cells in Tumor and Necrotic Fractions

Although viable tumor and necrosis clearly can be divided macroscopically, it is obvious that a perfect separation of pure tumor and necrotic material is not possible. Histological examination of the tumors of the first group (plus two extra) and two tumors of the second group was performed; it was revealed that ~70% (first group) of the tumor fraction consisted of viable tumor cells and 30% of the necrotic fraction. For the two tumors of the second group, these percentages were in the same range.

Time Dependence of 5-FU Metabolite Pattern in Tumor and Necrotic Tissue

To determine the time course of 5-FU metabolism in viable and necrotic tumor tissues, 20 days after transplantation, when the tumors had reached a mean total weight of 1.8 g, tumor extracts from both parts obtained at various time intervals after 5-FU administration were analyzed by 19F NMR. The results of these experiments are presented in Fig. 2. Figure 2 (a–d) shows the absolute concentrations of A, C, F, and T as observed at the various time intervals. The results demonstrate that F increases rapidly within the first 15 min, especially in the viable tumor part, to decrease thereafter. A and C increase in a similar parallel way up from 0 (time of 5-FU injection) to ~70 min. After an initial increase and a smaller decline thereafter, T stays at a stable level for the first 2 h. The tendencies are the same for the viable and necrotic tumor part. In the latter, the absolute values, except the concentration of F, are lower most of the time. This is particularly the case for the period after 50 min.

DISCUSSION

Only a few 19F NMR extract studies of 5-FU-treated tumors have been reported (4-6, 18, 19). Most of the 19F NMR studies on 5-FU metabolism in animals or man have been conducted in vivo. In addition, 19F NMR analysis has been performed in cell cultures (20). The role of necrosis in the interpretation of results of 5-FU metabolism has not been studied yet by 19F NMR. Because 5-FU metabolite levels may be quite different in necrotic parts, compared with viable parts of a tumor, the necrotic fraction may contribute significantly to the liabilities. Metabolite levels may be quite different in necrotic parts, compared with viable parts of a tumor, the necrotic fraction may contribute significantly to the liabilities. Metabolite levels may be quite different in necrotic parts, compared with viable parts of a tumor, the necrotic fraction may contribute significantly to the liabilities. Metabolite levels may be quite different in necrotic parts, compared with viable parts of a tumor, the necrotic fraction may contribute significantly to the liabilities.

In the necrotic fraction of the tumor, the absolute concentrations of all metabolites were lower than in the viable tumor fraction. The initial higher increase of 5-FU in viable tumor parts, compared with necrotic parts, most likely is caused by better vascularization of viable tumor.
tissue. After ~30 min, however, 5-FU levels become similar in both parts. Various processes may contribute to this behavior. Diffusion of 5-FU into necrotic tissue will occur, but at the same time 5-FU is better cleared from the well-vascularized, viable part of the tumor. Another process may be acidification of the viable cells in the necrotic part, inducing local retention of 5-FU (21). Whatever the responsible mechanism may be, the results indicate that 5-FU permeates into the necrotic area to a similar level as in the viable tumor area.

Anabolite levels are higher in tumor parts than in necrotic parts, which could be expected on the basis of the metabolic activity in viable tumor cells. Diffusion processes apparently are not able to equalize anabolite levels among viable and necrotic tumor parts. Considering the amount of viable tumor cells still present in the necrotic fraction, it is conceivable that an appreciable part of the anabolites in this fraction arise from these cells. In this tumor model, the amount of necrosis seems not of major importance for the response to therapy, because 5-FU reaches also the necrotic fraction wherein the viable cells anabolites can be formed.

Because catabolites are produced predominantly by detoxification of 5-FU in the liver, the well-vascularized part of the tumor will be supplied by increasing amounts of these catabolites, as is observed in this study. The lower levels of catabolites in the necrotic part of the tumor are likely caused by bad vascularization, which is not sufficiently compensated by diffusion from viable tumor parts.

It is common practice in animal studies to take tumors not exceeding 10% of body weight. This because of the possible occurrence of anemia influencing tumor perfusion (viscosity and oxygenation, and functional compromise by the tumor burden (22). As a result, substantial necrosis may develop. From this study, it follows that, in a tumor with a significant amount of necrosis, the conversion from 5-FU to anabolites and catabolites may be underestimated, as well as the A/C ratio. Therefore, it is of importance in 5-FU metabolism studies either to limit the amount of necrosis or to consider a possible contribution of necrosis.

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