EFFECT OF LEUCOVORIN ON 5-FLUOROURACIL INDUCED INHIBITION OF THYMIDYLATE SYNTHASE IN PATIENTS WITH COLON CANCER

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INTRODUCTION

5-Fluorouracil (5FU) is often used in the treatment of patients with advanced colorectal cancer [1], but most tumors show intrinsic or acquired resistance; the objective response rates are below 20%. Currently leucovorin (LV; 5-formyl-tetrahydrofolate) is added to this therapy leading to improved response rates generally between 30-40% [2]. Resistance against 5FU in patients may be related to a reduced inhibition of thymidylate synthase (TS) by FdUMP, the active metabolite of 5FU [1]. TS catalyzes the conversion of dUMP to TMP for which 5,10-methylene-tetrahydrofolate (CH_2-THF) is the methyldonor. Inhibition of TS by FdUMP is mediated by the formation of a covalent ternary complex between FdUMP, TS and CH_2-THF. The extent and retention of inhibition of TS, depending on the concentrations of FdUMP and CH_2THF, are considered to be limiting factors. In addition, TS can have different characteristics in tumors of different patients, determining whether inhibition by FdUMP is effective or not. It has been demonstrated by others [3,4] and ourselves [5] that TS shows a very large heterogeneity in colorectal cancer; this includes a very large variation in enzyme activity (more than 20-fold) and variations in kinetic properties (affinity of FdUMP and of the natural substrate dUMP for TS). Also different affinities for the folate-cofactor have been demonstrated [6]. Spears et al [7] postulated that a number of these factors may determine resistance to inhibition by FdUMP. Several authors determined inhibition of TS in tumors of patients after treatment with 5FU [7-9]. However, generally the time of biopsies was limited to a few hr after 5FU administration, but never longer than 24 hr. Since a long retention may be a crucial factor in determining the antitumor effect we measured the inhibition of TS in tumors and normal tissues of patients 48 hr after treatment with 5FU. In addition we determined whether enhancement of the intra-tumoral folates pools by administration of LV would lead to a more pronounced inhibition of TS. We not only measured TS with the FdUMP ligand bindings assay but also with the catalytic assay, i.e. the conversion of dUMP to TMP.

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MATERIALS AND METHODS

Patients Only patients with histologically proven colorectal cancer (both primary and metastasized), were entered into the study. Biopsy specimens from 19 patients (9 males/10 females; median age 57, range 37-72) were obtained after about 2 days (range 40-49 hr) after treatment. All patients were treated with an i.v. injection of 5FU at 500 mg/m^2, and 11 patients received 5FU in the middle of a 2-hr infusion of LV (500 mg/m^2). Tissues were frozen in liquid nitrogen as soon as possible after surgical removal, dissected and stored at -80^{O}C until analysis. Before analysis tissues were pulverized using a micro-dismembrator [10].

Enzyme assays Since no pretreatment samples could be obtained, the amount of TS inhibition had to be evaluated through dissociation of FdUMP from the ternary complex; after this process the total number of FdUMP binding sites (TS-*tot*) and the total TS catalytic activity (TS-*total*) could be determined. The uninhibited activity in the non-dissociated samples was termed TS-*free* (remaining number of FdUMP binding sites) and TS-*res* (residual catalytic TS activity). After pulverization of the tissues the frozen powder was suspended in the "sonication" buffer (pH 7.4) and centrifuged twice (400 and 7000g). Of the 7,000 g supernatant one part (for TS-*free* and TS-*res*) was frozen at -80°C and to the other part "dissociation" buffer (pH 7.8 containing CMP, NaF and excess dUMP) was added and incubated at 30°C for 3 hr [11]. Nucleotides were removed by a neutral charcoal wash [11]. In both parts we measured FdUMP binding and TS catalytic activity. TS-*tot* and TS-*free*, the amount of free FdUMP binding sites which can be bound by [6-^{3}H]-FdUMP, were determined according to a modification [5,11] of published methods [7]. TS-*total* and TS-*res* were measured with the tritium release assay [5]. The catalytic TS activity was determined at 1 and 10 μM dUMP, being suboptimal (K*m*) and saturating dUMP concentrations. As discussed elsewhere evaluation of TS inhibition can sometimes be hampered by the experimental conditions [11]. Optimization and standardization minimized variation; dUMP was added to optimize dissociation and stabilization of the enzyme; NaF and CMP were added to inhibit phosphatase and 5'-nucleotidase mediated nucleotide degradation; the assay time for the bindings assay was limited to minimize exchange of labeled and non-labeled FdUMP. Tissues were considered as evaluable when the radioactivity was at least 1.5 *x* higher than in the blank.

RESULTS

In tissue biopsies (primary tumors, liver metastases, colon mucosa and liver) of patients we studied several characteristics of TS using the ligand binding assay for FdUMP and the catalytic assay for TS. The total activity of TS (both FdUMP binding and catalytic activity) in tumors and metastases obtained after 48 hr, showed a large variation, and was generally higher (Table 1) than in normal mucosa and liver from the same patient. Because of the higher sensitivity of the catalytic assay more samples could be evaluated with this assay than with the FdUMP binding assay, this difference was more pronounced in samples of the liver and normal mucosa. In addition the sensitivity of the catalytic assay at a substrate concentration of 1 μM was higher than at 10 μM. The ratio between the activity at 10 and 1 μM dUMP varied between 2 and 8. From 4 patients both primary tumors and liver metastases could be obtained. Since no significant difference was observed in values obtained from patients treated with either 5FU alone or with the combination of LV and 5FU, these values have been pooled (Table 1).

Table 1. FdUMP Binding (TS-*tot*) and Total Catalytic TS Activity (TS-*total*); effect of 5FU and LV/5FU treatment in tumors.

Parameter	TS-*tot* and TS-*total*			Ratio of TS-*free/tot* and TS-*res/total* in tumors	
	Mucosa	Liver	Tumor	5FU	LV-5FU
FdUMP binding	0 (24)	21 (21)	83 (24)	0.37 [6]	0.24 [12]
Catalytic activity					
1 μM dUMP	26 (4)	5 (14)	34 (24)	0.68 [8]	0.49 [12]
10 μM dUMP	105 (4)	25 (12)	129 (22)	0.59 [7]	0.31 [13]

Values (from tissues removed 2 days after treatment) for TS-*tot* (fmol/mg protein) and TS-*total* (pmol/hr/mg protein) represent medians of the number of tissues indicated within ( ). Values below detection limit (< 1.5 *x* blank) are denoted as 0. From several patients both primary tumors and metastases were obtained. Ratios were calculated from the separate tumors and represent means of the number of evaluable tumors indicated within [ ].

Inhibition of TS was evaluated by comparison of the TS-*free* and TS-*res* among the patients with TS-*tot* and TS-*total*, respectively. Ratios could be only calculated in those patients in which both assays were evaluable. In tumors from patients treated with 5FU alone the ratios were higher than in tumors from patients treated with LV-5FU. In normal liver no difference between 5FU and LV-5FU treatment was observed. Several patients were subsequently treated with intra-arterial 5FU administration (1000 mg/m^2/day for 5 days) and the total activity of TS and the inhibition of TS could be related to the outcome of therapy. In 4 patients from the 5FU pretreated group with a low TS-*free* and TS-*res* (below or at detection limit) disease stabilization (2 *x*) and a partial response (2 *x*) were observed; from the LV/5FU pretreated group 3 patients with such a low TS activity had a disease stabilization after subsequent 5FU therapy. In one patient with high TS values progression was observed. The other patients were not yet evaluable for response.

DISCUSSION

In this study we demonstrate that the TS activity in tumors is still inhibited up to 2 days after treatment with single agent 5FU. In patients treated with the combination of LV and 5FU this inhibition is more pronounced. In other studies evaluation of the inhibition of TS in tumors from patients has been limited to shorter time periods using the FdUMP bindings assay [7-9]. Swain et al [8] demonstrated that a partial TS inhibition in patients with 5FU-resistant breast cancer could be converted to a complete inhibition after LV-5FU treatment. In addition to the FdUMP bindings assay used by others we also evaluated TS inhibition using the catalytic assay. The assay appeared to be more sensitive than the bindings assay and gives an estimate of the capacity of tumors to convert dUMP to TMP. Since inhibition of TS can lead to an increase in dUMP levels [7], the use of low and high substrate concentrations gives some insight in the potential inhibition of the enzyme in the tumor. Relative inhibition of TS at 10 μM dUMP concentration was usually higher than at low dUMP.

An attempt was made to correlate the inhibition of TS with the response to subsequent 5FU treatment. The present data and evaluation of TS inhibition at shorter time periods [11] demonstrate that a complete inhibition of TS at 24 or 48 hr after 5FU treatment is likely to be related with effective 5FU treatment (partial response and disease stabilization). In addition high TS-*tot* or TS-*total* values have been associated with ineffective treatment. The activity of TS after dissociation is considered to represent pretreatment TS levels, so, evaluation of TS in patients before treatment might give indication whether subsequent treatment will be succesful [5]. In addition to enzyme measurements TS can also be evaluated by determining the amount of TS protein using specific antibodies [12] and by determining the expression of TS-mRNA using PCR [13]. Both approaches are currently being evaluated.

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