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ORIGINAL ARTICLE

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Does cisplatin (CDDP) function as a modulator of 5-fluorouracil (5-FU) antitumor action? A study based on a clinical trial

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Abstract *Objective*: To clarify whether CDDP acts as a modulator of 5-FU antitumor action in gastric cancer, patients were treated preoperatively with 5-FU + CDDP (FP) chemotherapy. *Patients and methods*: From September 2000 to November 2001 at Takarazuka Municipal Hospital, 29 patients preoperatively diagnosed with stages II–IV gastric cancer were enrolled. Written informed consent was obtained from all patients. The patients were randomly assigned to two groups: the FU group, in which patients received a continuous intravenous infusion of 5-FU 320 mg/m^2 per day over 24 h a day for 5 days beginning 5 days prior to surgery, and the FP group, in which patients received bolus intravenous injections of CDDP 3.5 mg/m^2 per day for 5 days prior to surgery in addition to the same infusion of 5-FU as the FU group. As indicators of the intracellular effect of 5-FU treatment, thymidylate synthase (TS) inhibition rates, TS protein levels, TS and dihydropyrimidine dehydrogenase (DPD) activity, and F-RNA concentrations were measured. *Results*: Using Scheffe's multiple comparison test, in both treatment groups the tumor regions were found to have significantly higher TS inhibition rates than the nontumor regions ($P < 0.05$). No significant differences in TS protein levels, TS activity, DPD activity or F-RNA concentrations were found between the four regions. *Conclusions*: Our results show that CDDP clinically may act to enhance the antitumor effects of 5-FU in terms of the inhibition of DNA synthesis and could therefore act as a modulator of 5-FU.

Keywords Gastric cancer · 5-Fluorouracil · Inhibition rates of thymidylate synthase

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Introduction

Biochemical modulation is the alteration of the antitumor actions of an effector drug through modification of metabolic pathways by the action of another drug (the modulator) [1]. At least two mechanisms of the antitumor action of 5-FU have been suggested. One involves the inhibition of DNA synthesis through thymidylate synthase (TS) inhibition forming ternary complexes with one of the active 5-FU metabolites, fluorodeoxyuridine monophosphate (FdUMP), and the reduced form of folic acid, 5,10-methylene tetrahydrofolate (5,10-CH_2-FH_4) [2–6]. The other involves the inhibition of RNA function through the incorporation of another active 5-FU metabolite, fluorouridine triphosphate (FUTP), into the RNA resulting in the creation of fraudulent-RNA (F-RNA) [7–11]. Because the amounts of reduced forms of folic acid stored within the tumors is low, there have been cases reported in which the antitumor action of 5-FU has been found to be insufficient [12]. It is therefore believed that a modulator acting to increase the concentration of the reduced forms of folic acid would be useful in increasing the antitumor effects of 5-FU. Excellent results with 5-FU + CDDP (FP) chemotherapy in gastric cancer have been reported [13–17]. The mechanisms of the antitumor action of CDDP are thought to be related mainly to the direct binding of two guanine bases to the DNA strand and inhibition of DNA synthesis [18]. On the other hand, it is also thought that CDDP acts to increase the concentrations

of reduced forms of folic acid within the tumor. The mechanisms for this are thought to be related to decreases in the intracellular concentrations of methionine due to inhibition of its influx via the extracellular membrane as a result of the action of CDDP, and the biosynthesis of methionine from homocysteine which is promoted to compensate for the decreasing levels of intracellular methionine. In connection with this pathway, the biosynthesis of intracellular reduced forms of the folic acids FH_4 and 5,10-CH_2FH_4 are increased. In vitro and in vivo studies [19–21] have shown that the administration of 5-FU causes the formation of ternary complexes to levels sufficient for the effective inhibition of TS activity and enhancement of the antitumor effect of 5-FU. However, it is not clear whether CDDP acts clinically as a modulator of 5-FU by such mechanisms. In this study, we sought to determine clinically whether CDDP acts as a modulator of 5-FU by measuring the TS inhibition rates, TS protein levels, TS activity, DPD activity and F-RNA concentrations in resected specimens from patients with gastric cancer undergoing preoperative 5-FU or FP chemotherapy.

Patients and methods

Enrolled in the study were 29 patients admitted to Takarazuka Municipal Hospital from September 2000 to November 2001 preoperatively diagnosed with stages II–IV gastric cancers. Written informed consent was obtained from all patients. Patients were randomly assigned to two groups for preoperative chemotherapy treatments: the FU group, in which patients received 5-FU 320 mg/m^2 per day as a continuous intravenous infusion for 24 h a day over a period of 5 days beginning 5 days prior to surgery, and the FP group, in which patients received bolus intravenous injections of CDDP 3.5 mg/m^2 per day for 5 days prior to surgery in addition to the same infusion of 5-FU as the FU group. Surgically resected specimens were immediately separated into tumor and nontumor regions. The nontumor regions were defined as the region located approximately 5 cm away from the rim of the tumor with cells histopathologically not exhibiting characteristics of tumor cells. At least 1 g of the tumor and nontumor regions were individually obtained and were kept in a freezer at −80°C until use.

TS inhibition rates, TS protein levels, TS activity, dihydropyrimidine dehydrogenase (DPD) activity, and F-RNA (or 5-FU-incorporated RNA) concentrations were measured as indicators of the intracellular effect of 5-FU treatments.

Measurement of protein levels and inhibition rates of TS (radiobinding assay method)

TS protein levels (FdUMP-bound and FdUMP-unbound TS levels in the supernatant, TS_{total}, and FdUMP-unbound TS levels in the supernatant, TS_{free}) were measured according to the method described by Spears et al. [4] with slight modification (the homogenizing buffer and dissociation buffer were changed to 50 mmol/l phosphate buffer, pH 7.4, and 50 mmol/l Tris buffer, pH 8.0, respectively). For the determination of TS_{total} levels, the supernatant was incubated at 25°C with an equal volume of 50 mmol/l Tris buffer (pH 8.0) in order to dissociate TS from the complex contained in the supernatant. After addition of [^{3}H]-FdUMP and methylene tetrahydrofolate (mTHF), the mixture was incubated at 25°C again to allow a ternary complex containing [^{3}H]-FdUMP to form. The complex was isolated and the amount of radioactivity determined. For the determination of TS_{free} levels, an equal volume of 50 mmol/l Tris buffer was added to the supernatant, followed by the addition of [^{3}H]-FdUMP and mTHF, and the mixture was incubated at 25°C to allow the complex to form. The complex was isolated and the amount of radioactivity determined in a similar manner to the TS_{total} assay. From the TS_{total} and TS_{free} levels, the TS inhibition rates can be calculated using the following equation:

$$\text{TS inhibition rates}\,(\%) = [1 - TS_{free}/TS_{total}] \times 100$$

Measurement of TS activity in tissue (tritium release method)

TS activity was measured according to the method described by Etienne et al. [22] with slight modification (the concentration of substrates and proportion of reaction mixture were changed). After centrifugation (105,000 *g*, 1 h, 4°C) of the tissue homogenate, the supernatant was incubated at 37°C with methylene tetrahydrofolate and [^{3}H]-dUMP as the substrate for 30 min. From the reaction rate and the protein concentration determined separately, TS activity (picomoles per minute per milligram protein) was calculated.

Measurement of DPD activity in tissue (RI-HPLC method)

DPD activity was measured according to the method described by Diasio et al. [23] with slight modification (the concentration of substrates and proportion of reaction mixture were changed). After centrifugation (105,000 *g*, 1 h, 4°C) of the tissue homogenate, the supernatant was incubated at 37°C in the presence of 6.25 mmol/l NADPH and 125 μmol/l [^{3}H]5-FU (25 μCi/ml) for 30 min. Then the supernatant obtained was analyzed by the RI-HPLC procedure using a YMC-Pack Pro C18 column (AS-301-3, 4.6×100 mm; YMC, Kyoto, Japan) and a mobile phase comprising 20 mmol/l phosphate buffer (pH 3.5). The reaction rate was obtained based on the relationship between reaction time and the total concentrations of 5-FU and its

Table 1 Patients' characteristics, histological response and side effects

	FU group	FP group	*p* value
Age	66 (46–81)	63 (40 ∽ 76)	*0.359*[#]
Sex (male/female)	8/5	13/3	*0.406*[§]
Histology			
Well diff.	0	1	
Moderately diff.	5	9	
Poorly diff.	3	2	*0.699*[¶]
Mucinous	1	1	
Signet ring cell ca.	4	3	
Postsurgical stage classification			
Stage I	7	8	
Stage II	0	5	*0.602*[$]
Stage III	4	4	
Stage IV	2	0	
Vessel invasion (v)			
v(–)	5	4	*0.688*[§]
v(+)	8	12	
Lymphatic invasion (ly)			
ly(–)	2	3	*1.000*[§]
ly(+)	11	13	
Histological response			
Grade 0	13	15	
Grade 1a	0	1	*1.000*[§]
Grade 1b	0	0	
Grade 2	0	0	
Grade 3	0	0	
Side effects (≧Grade 1)			
Preoperative side effects			
Anorexia	0/13 (0%)	0/16 (0%)	–
Leukopenia	0/13 (0%)	0/16 (0%)	–
Thrombocytopenia	0/13 (0%)	0/16 (0%)	–
AST (SGOT)	0/13 (0%)	0/16 (0%)	–
Postsurgical complications			
Suture insufficiency	0/13 (0%)	1/16 (6%)	*1.000*[§]
MRSA infection	0/13 (0%)	0/16 (0%)	–

[#]Student's t-test, [§]Fisher's exact probability test, [¶]χ2-test, [$]U-test

metabolites, 5-FDHU, FUPA and FBAL. From the reaction rate and the protein concentration determined separately, DPD activity (picomoles per minute per milligram protein) was calculated.

Measuring 5-FU concentrations within the tissue RNA (GC-MS method)

F-RNA concentration was measured according to the method described by Masuike et al. [24], but with alteration of trimethylsilylation of 5-FU to benzylation with 3.5-di(trifluoromethyl)benzyl bromide. The derivatized 5-FU was analyzed by GC-MS.

From the RNA concentrations obtained and the results of GC-MS using a DB-1 fused silica capillary column (0.25 mm×30 m; J&W Scientific, Folsom, Calif.), and 5-FU *m*/*z* 355 and IS *m*/*z* 357 monitor ions, the amount of 5-FU incorporated into RNA (nanograms per milligram RNA) was calculated.

These indicators were determined for the FU tumor and nontumor regions and the FP tumor and nontumor regions. Measured values are expressed as means ± SE. Levels of significance were determined using Student's paired and unpaired *t*-tests, Fisher's exact probability test, χ^2-test, *U*-test and Scheffe's multiple comparison. *P* values less than 0.05 were considered to indicate statistical significance.

Results

Patient characteristics, histological response and side effects

Enrolled in the study were 29 patients with gastric cancer. Table 1 presents the characteristics of these patients; no significant differences were observed in the characteristics between the two treatment groups. The histological effect was evaluated based on grade according to the criteria of the Japanese Research Society for Gastric Cancer: *grade 0* no histological change; *grade 1a* slight change—necrosis or disappearance of tumor in less than one-third of the entire lesion, or only cellular or structural changes; *grade 1b* slight change—necrosis or disappearance of tumor in no more than two-thirds of the entire lesion; *grade 2* moderate change—necrosis or disappearance of tumor in more than two-thirds of the entire lesion, but viable tumor cells still remaining; and *grade 3* marked change—whole lesion necrotic and/or replaced by fibrosis, with or without granulomas, no viable tumor cells observed. In the FU group 13/13

(100%) were grade 0, and in the FP group 15/16 (93.7%) were grade 0 and 1/16 (6.3%) was grade 1a. Grades 1b, 2 and 3 were not observed in either group. Preoperative side effects (grade 1 or more anorexia, leukopenia, thrombocytopenia, AST (SGOT), according to the criteria of NCI-CTC, version 2.0) were not observed in either group. Postsurgical MRSA infection was not occurred in either group, but suture insufficiency (dermatology/skin-other grade 1; mild) occurred in one patient in the FP group.

TS inhibition rates

The TS inhibition rates in the tumor and nontumor regions in the FU group were $54.80 \pm 3.27\%$ and $33.83 \pm 4.51\%$, respectively, and in the FP group were $82.46 \pm 3.48\%$ and $35.78 \pm 6.79\%$. Scheffe's multiple comparison indicated that the differences in each group were significant ($P < 0.05$).

The TS inhibition rate of the FP tumor specimens was significantly higher in comparison with the TS inhibition rates of the other three groups, namely the FP nontumor ($P < 0.001$), FU tumor ($P = 0.027$) and FU nontumor ($P < 0.001$) specimens. There were no significant differences observed between the FU tumor group and the FU nontumor group ($P = 0.146$; Fig. 1).

Amounts of TS protein (TS_{total})

The amounts of TS protein in the tumor and nontumor regions in the FU group were 5.48 ± 1.22 and 3.98 ± 0.37 pmol/mg protein, respectively, and in the FP group were 9.43 ± 3.35 and 2.78 ± 0.40 pmol/mg protein, respectively. Scheffe's multiple comparison revealed no significant differences between the four regions.

TS activity

The TS activities in the tumor and nontumor regions in the FU group were 4.28 ± 1.21 and 2.95 ± 0.43 pmol/min/mg protein, respectively, and in the FP group were 6.23 ± 1.95 and 2.25 ± 0.46 pmol/min/mg protein, respectively. Scheffe's multiple comparison revealed no significant differences between the four regions.

DPD activity

The DPD activities in the tumor and nontumor regions in the FU group were 59.1 ± 12.6 and 37.8 ± 6.3 pmol/min/mg protein, respectively, and in the FP group were 32.2 ± 6.1 and 25.4 ± 3.5 pmol/min/mg protein, respectively. Scheffe's multiple comparison revealed no significant differences between the four regions.

F-RNA concentration

The F-RNA contents in the tumor and nontumor regions in the FU group were 56.1 ± 14.0 and 29.4 ± 4.5 ng/mg RNA, respectively, and in the FP group were 43.0 ± 8.1 and 25.1 ± 4.5 ng/mg RNA, respectively. Scheffe's multiple comparison revealed no significant differences between the four regions.

Discussion

TS inhibition rates were measured as an indicator of the DNA synthesis inhibitory action of 5-FU. TS inhibition rates were significantly different between the FP group tumor region and the FP nontumor, FU tumor and FU nontumor regions. F-RNA concentrations were measured as an indicator of RNA dysfunction. However, we

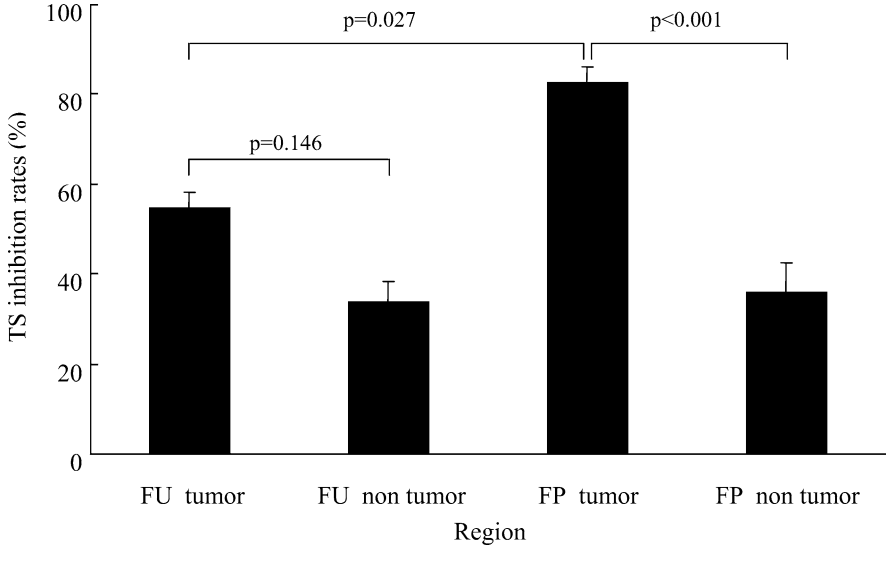

Fig. 1 TS inhibition rates

found no significant differences between the four regions. From these results, we consider that CDDP may act as a modulator of 5-FU DNA synthesis inhibition especially in tumor regions. It is thought that the high expression levels of TS protein as well as insufficient amounts of reduced forms of folic acid and the supply of FdUMP are factors contributing to the incomplete inhibition of TS. Using human gastric cancer tissue, Dohden et al. [25] have shown that there is a negative correlation between the amounts of TS protein within tumor tissue and the TS inhibition rates obtained when sufficient amounts of FdUMP are supplied. Also, insufficient supplies of FdUMP are the result of metabolism of 5-FU by DPD. In tumor regions, no significant differences were observed between the FU group and FP group with respect to either the levels of TS protein or DPD activity. We therefore consider that there were no differences in TS protein and FdUMP supplies.

Although the concentrations of the reduced forms of folic acid were not measured, the differences observed in the TS inhibition rates especially in the tumor regions in the FU and FP groups can be considered to be due to increased concentrations of the reduced forms of folic acid as a result of the presence of CDDP. Shirasaka et al. [21] have reported that in vivo CDDP acts to increase the antitumor effects of 5-FU by increasing the concentrations of reduced forms of folic acid within the tumor tissue. Our clinical results support this result. In the present study, we tried to evaluate whether the extent of the histological changes as a secondary endpoint could be used as an indicator for assessing the clinical effects. However, the modulating effects of CDDP on 5-FU were not confirmed from the data obtained during a 5-day observation period.

There are time lags among the various cellular responses after administration of 5-FU. More specifically, TS inhibition, as well as the changes in cell cycle, occur quite early and are considered early responses, whereas cellular degeneration and tumor growth suppression are considered delayed responses. Yamane et al. [26] have reported, following a study of preoperative 5-FU in patients with gastric cancer, that the changes in the cell cycle distribution are maximized with a 5-day preoperative chemotherapy, whereas the apoptotic index, based on a positive rate in the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay and the level of growth promotion based on the Ki-67-positive rates, is maximized with a 7-day preoperative chemotherapy.

Although a correlation between TS inhibition rate and prognosis could not be established in the present study due to the short postoperative observation period, Nakano et al. [27] have reported that the 1-year survival rate is significantly related to the TS inhibition rate, following a study of preoperative chemotherapy with 5-FU in patients with advanced gastric cancer investigating the relationship between TS inhibition rate and prognosis. The measurement of TS inhibition rate may be useful as a prognostic factor in determining the need for postoperative adjuvant chemotherapy or even in selecting medical regimens, since significantly higher TS inhibition rates were obtained in the FP group than in the 5-FU group. However, we believe it is too early to conclude this based solely on our data at this time. The correlation between TS inhibition rate and the clinical effects will be further investigated through continuous follow-ups.

Although no significant differences were observed between the FU and FP groups in the correlation between the incidence of side effects and TS inhibition rate in normal tissue ($P = 0.835$), we cannot conclude that there were no differences between the FU and FP groups based only on the data from this observation period considering the timing of the onset of side effects. In the present study, there were no side effects observed, except in one patient in the FP group who underwent highly invasive surgery involving hepatectomy and had postoperative failure of the sutures but with no histological changes. We believe that this incident was not related to preoperative chemotherapy, or more specifically to the chemotherapy with FP. In addition, 5-FU with very low dose concurrent and sequential CDDP as used in our study has only been evaluated in a small phase II study. In future, we need to evaluate its efficacy by comparing the data from 5-FU with conventional single-dose CDDP in a large number of patients to further investigate the effects of TS inhibition rate as well as its association with tumor suppression, prognosis and side effects.

In this study, because it was difficult to obtain specimens of sufficient volume before preoperative chemotherapy, we could not measure the prechemotherapeutic levels of TS activity and TS protein. With regard to TS activity, we found no significant differences among the four regions; however, these results were obtained after the administration of anticancer drugs. It has been reported that some anticancer drugs act to modify TS activity as well as the levels of TS protein. Because of this, it is necessary to consider results after measuring the levels of TS activity and TS protein both before and after the administration of the anticancer drugs. Although it is more useful to measure mRNA contents than enzyme activities in terms of sample amounts, the relationships between these measurement are still unclear. So further studies are needed to confirm the utility of the measurement of mRNA content in the assessment of the biochemical modulation of 5-FU.

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