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Synergistic Effect of Methionine-depleting Total Parenteral Nutrition with 5-Fluorouracil on Human Gastric Cancer: A Randomized, Prospective Clinical Trial

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Methionine-depleting total parenteral nutrition (Met-depleting TPN), infusing AO-90 amino acid solution (lacking both L-methionine and L-cysteine) as a sole nitrogen source, showed synergistic effects with 5-fluorouracil (5-FU) in tumor-bearing rats and in clinical trials with gastrointestinal tract cancers. In this study, the effect of Met-depleting TPN with 5-FU upon thymidylate synthase (TS) activity was examined, and the histological effect of this treatment on human gastric cancer was evaluated. Fourteen preoperative advanced gastric cancer patients were divided randomly into two groups. Seven cases were given Met-depleting TPN for 7 days before surgery with continuous intravenous administration of 5-FU (500 mg/body per day; total 4.0 g/body) (AO-90 group). The other 7 received conventional L-methionine-containing TPN with 5-FU (control group). All patients underwent gastrectomy without complications due to these treatments. Resected materials were examined for TS kinetics, and the anti-cancer effect was also assessed histopathologically. The specimens in the AO-90 group showed marked degeneration of cancer, while almost no effect was seen in the control group. The free TS activity of carcinoma tissue in the AO-90 group was decreased and the TS inhibition rate was increased in comparison with the control group ($P=0.0165$ and $P=0.0243$, respectively). Met-depleting TPN appears to play a role as a biomodulator of 5-FU in human gastric cancer.

Key words: Methionine-depleting TPN — 5-FU — Thymidylate synthase inhibition — Gastric carcinoma — Histopathological grade of anticancer effect

Met-depleting TPN,[6] infusing AO-90 amino acid solution devoid of all sulfur-containing amino acids as the only nitrogen source, has enhanced the anti-tumor effect of several agents in animal experiments and clinical trials.[1–12] Notably, the anti-cancer effect of 5-FU was enhanced by this parenteral treatment, not only in tumor-bearing animal experiments but also in clinical trials with gastrointestinal tract cancers.[4,6,9,11,12] One of the mechanisms of this synergistic effect with 5-FU is thought to be so-called biochemical modulation.[13] In Sato lung carcinoma-bearing rats, L-methionine concentration in the tumor tissue was markedly reduced by Met-depleting TPN compared to the L-methionine-infused control group.[7] In YS-bearing rats, both folic acid and methylene tetrahydrofolate in tumor tissue tended to increase and TSIR was increased by this parenteral treatment with 5-FU.[14] It was considered that DNA synthesis might have been inhibited, which would result in tumor growth inhibition.

Therefore, we performed a randomized prospective trial to examine whether or not such a phenomenon could also be identified in human gastric carcinomas by histological observation of the anti-cancer effect. The free and total TS activities in both carcinoma and cancer-free gastric mucosal tissues following preoperative Met-depleting TPN treatment with continuous 5-FU administration were compared with those in the case of the conventional TPN, infusing L-methionine, and 5-FU. A detailed histopathological examination of the resected materials was also conducted to compare the anti-cancer effect in the two groups.

PATIENTS AND METHODS

Study subjects For this study, we selected 14 advanced gastric cancer patients with stenosis or obstruction of the

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[6] The abbreviations used are: Met-depleting TPN, methionine-depleting total parenteral nutrition; TPN, total parenteral nutrition; 5-FU, 5-fluorouracil; YS, Yoshida sarcoma; TSIR, thymidylate synthase inhibition rate; DNA, deoxyribonucleic acid; TS, thymidylate synthase; TS-free, free thymidylate synthase; TS-total, total thymidylate synthase; CH_2FH_4, L-(+)-5,10-methylenetetrahydrofolate; FdUMP, tritiated 5-fluoro-2′-deoxyuridylate; MS1, N^5-methyltetrahydrofolate-homocysteine methyltransferase; MS2, betaine-homocysteine methyltransferase; FH_4, tetrahydrofolic acid; BSA, bovine serum albumin.

gastric canal due to cancer infiltration from 53 consecutive gastric carcinoma patients admitted to the First Department of Surgery, Tokyo Medical and Dental University School of Medicine, and the Department of Surgery, Edogawa Hospital, between June 1991 and January 1992. They were randomly allocated to two groups by sealed envelope randomization; the AO-90 group and the control group. This research protocol was approved by the Medical Research and Development Command at Tokyo Medical and Dental University. All patients gave informed consent.

Table I summarizes the clinico-pathological findings in the 14 cases such as age, sex, macroscopic findings and staging of gastric cancer bsed on the General Rules for the Gastric Cancer Study by the Japanese Research Society for Gastric Cancer.[15] The AO-90 group consisted of 4 males and 3 females, aged from 55 to 78 (average 67.1); 3 were stage IV and 4 were stage III. The control group included 6 males and 1 female, aged 49 to 75 (average 62.4); 5 were stage IV and 2 were stage III.

Protocol of preoperative TPN treatments All patients received TPN with the intravenous administration of 5-FU at a daily dose of 500 mg/body for 24 consecutive hours (total 4.0 g/body) from 7 days before operation to completion of gastrectomy. In the AO-90 group, AO-90 amino acid solution was given as a sole nitrogen source with glucose, lipid, electrolytes, vitamins and minerals. The control group received commercial Proteamine 12X (Tanabe Pharmaceutical Co., Ltd., Tokyo), an amino acid solution containing L-methionine and other nutrients.

Table II lists the components of AO-90 and Proteamine 12X amino acid solutions. Table III summarizes the daily TPN components infused into both groups. All patients ate nothing in order to prevent protein intake during the TPN treatment, and underwent gastrectomy in the 8th day of the TPN treatment. 5-FU was adminis-

Table I. Clinico-pathological Findings

Group	Case No.	Sex	Age (yr)	Stage[a]	Macroscopic type[a]
AO-90 (n=7)	1	Female	63	III	5
	2	Male	58	III	4
	3	Male	68	IV	3
	4	Male	70	III	4
	5	Male	78	IV	2
	6	Female	55	IV	3
	7	Female	78	III	2
Control (n=7)	1	Male	75	IV	2
	2	Male	51	III	2
	3	Male	70	IV	3
	4	Female	61	IV	3
	5	Male	56	IV	3
	6	Male	49	III	3
	7	Male	75	IV	3

a) Classification according to the General Rules for the Gastric Cancer Study.[15]

Table II. Components of AO-90 and Proteamin 12X Amino Acid Solutions

Component	Amino acid solution	
	AO-90 (g/100 ml)	Proteamin 12X (g/100 ml)
L-Arginine	0.66	0.66
L-Histidine	0.30	0.30
L-Isoleucine	0.55	0.55
L-Leucine	1.23	1.23
L-Lysine	1.49	1.49
L-Methionine	—	0.71
L-Phenylalanine	0.87	0.87
L-Threonine	0.54	0.54
L-Tryptophan	0.18	0.18
L-Valine	0.61	0.61
Glycine	1.00	1.00
Total amino acid (g/100 ml)	7.43	8.14
Total N (g/100 ml)	1.19	1.26

Table III. Daily TPN Admixtures Infused into the AO-90 and Control Groups

	AO-90 group (amount/day)	Control group
AO-90 (ml)	500.0	0.0
Proteamin 12X (ml)[a]	0.0	500.0
Hicaliq-2 (ml)[b]	1400.0	1400.0
Intralipid 20% (ml)[c]	250.0	250.0
MVI[d]	1 vial	1 vial
10% NaCl injection (ml)	40.0	40.0
Carbohydrate (g)	350.0	350.0
Nitrogen (g)	5.95	6.30
Fat (g)	50.0	50.0
Na (mEq)	68.0	68.0
K (mEq)	60.0	60.0
Cl (mEq)	68.0	68.0
Ca (mEq)	16.9	16.9
Mg (mEq)	20.0	20.0
Phosphate (mEq)	9.6	9.6
Total volume (ml)	1940.0	1940.0
Non-protein calorie (kcal)	1548.6	1562.8
5-Fluorouracil (mg)[e]	500.0	500.0

a) 10% amino acid solution, Tanabe Pharmaceutical Co., Ltd., Tokyo.
b) 25% glucose solution with electrolytes, Terumo Co., Tokyo.
c) 20% fat emulsion, Otsuka Pharmaceutical Co., Ltd., Tokyo.
d) Multi-vitamin infusion, S.S. Pharmaceutical Co., Ltd., Tokyo.
e) Antitumor agent, Kyowa Hakko Kogyo Co., Ltd., Tokyo.

tered with each TPN solution until gastrectomy was completed. No fluids containing protein were given.

Tissue specimens and analysis of TS-free, TS-total and TSIR In twelve cases, 6 in the AO-90 group and 6 in the control group, sufficient cancer tissues and cancer-free gastric mucosa were sampled for TS assay. These materials were frozen in nitrogen liquid immediately after resection and kept at −80°C until TS activity was analyzed.

Assays of TS-free and TS-total activities, and calculation of TSIR TS levels were measured by a modification of Spear's method.[16] The tissues (300 mg) were homogenized under ice cooling with 2 ml of potassium dihydrogenphosphate buffer (50 m*M*, pH 7.4) containing sodium fluoride (100 m*M*), cytidine 5′-monophosphate (15 m*M*) and 2-mercaptoethanol (20 m*M*). After having been homogenized under sonication, the mixture was centrifuged at 14,500 rpm for 3 h. The resulting supernatant was employed as the assay sample.

TS-total level was measured as follows: A mixture of supernatant (50 ml) and ammonium bicarbonate buffer (50 ml: 0.3 *M*, pH 8.1) containing sodium fluoride (100 m*M*), cytidine 5′-monophosphate (15 m*M*) and 2-mercaptoethanol (20 m*M*) was incubated at 25°C for the dissociation of TS from the ternary complex of FdUMP. After 3 h, 6 pmol of ^{3}H-FdUMP in 50 ml of phosphate buffer (5 m*M*) and 25 ml of cofactor solution containing FH_4 (1 m*M*), ascorbic acid (16 m*M*) and formaldehyde (9 m*M*) were added to the mixture and the whole was incubated at 25°C for 20 min in order to form TS-FdUMP-CH_2FH_4 ternary complex. Then, 1 ml of aqueous suspension, acidified with hydrochloride, of 3% dextran-coated charcoal was added to the mixture to remove the unreacted ^{3}H-FdUMP. The supernatant was obtained by centrifugation at 3,000 rpm for 30 min and its radioactivity was measured with a liquid scintillation counter. TS-free levels were also determined by the same procedure, but without preincubation to dissociate endogenous ternary complex. The value of TSIR was calculated by use of the following equation.

$$\text{TSIR}(\%) = (\text{TS-total} - \text{TS-free})/\text{TS-total} \times 100$$

Histopathological examination of resected materials Using the resected materials of 14 cases, sections about 5 mm wide were cut from the center of the cancer, including adjacent gastric wall with no cancer invasion, and fixed with 10% formalin. These sections were embedded in paraffin and sliced to about 4 μm in thick. Hematoxylin-eosin staining, and Alcian blue and PAS double staining were employed.

The histological examination of cancer tissue to evaluate the therapeutic response was conducted according to the histological criteria of the Japanese Research Society for Gastric Cancer,[15] as follows. Grade 0: No effect; almost no treatment-induced tumor degeneration or necrosis is seen. Grade 1: Slight effect; (a) Very slight effect; degeneration or necrosis affects $<1/3$ of the cancer cells. (b) Slight effect; degeneration, necrosis, or liquefaction of 1/3–2/3 of the cancer cells. Grade 2: Moderate effect; marked degeneration, necrosis, liquefaction, or disappearance of more than two-thirds of the cancer cells. Grade 3: Marked effect; the entire tumor is necrotic or liquefied, has disappeared or has been replaced by granulation tissue or fibrosis.

Statistical analysis Two-tailed unpaired *t* tests were used to compare the activities of TS-free, TS-total and TSIR in the carcinomatous and cancer-free mucosal tissues between both experimental groups. Anti-tumor effects were compared between the two groups with the Mann-Whitney U-tests in terms of the incidence and severity of histological response.

RESULTS

None of the 14 patients showed any major complication, such as marked myelo-suppression, hypoproteinemia, liver dysfunction or anastomotic leakage, due to preoperative treatment.

TS-free and TS-total activities in gastric carcinoma tissue and cancer-free gastric mucosa Table IV shows the mean±SD values of TS-free and TS-total activities, and TSIR in cancer tissues and tumor-free gastric mucosa in 12 cases (6 in the AO-90 group and 6 in the control group). The TS-total activity of tumor tissue was

Table IV. TS-free and TS-total Activities, and TSIR in Gastric Carcinomatous and Cancer-free Mucosal Tissues in the AO-90 and the Control Groups

Group	Tumor tissue			Cancer-free gastric mucosa		
	TS-total (pmol/g tissue)	TS-free (pmol/g tissue)	TSIR (%)	TS-total (pmol/g tissue)	TS-free (pmol/g tissue)	TSIR (%)
AO-90 (n=6)	4.50±1.59	1.12±0.74[a)]	76.27±9.50[b)]	3.07±1.55	1.15±0.52	56.28±25.97
Control (n=6)	4.98±2.50	2.35±0.74	45.87±26.43	4.23±1.70	1.85±0.93	55.08±14.40

Values are presented as mean±SD.
Significantly different from the control group by the two-tailed unpaired *t* test: *a*) $P=0.0156$, *b*) $P=0.0243$.

Table V. Histological Evaluation of Anti-cancer Effect

Group	Case No.	Macroscopic type[a)]	Histological type[b)]	Grade of histological response[a)]
AO-90	1	5	por	2
(n=7)	2	4	sig	1-b
	3	3	tub2	1-a
	4	4	por	0
	5	2	tub1	2
	6	3	sig	2
	7	2	tub1	2
Control	1	2	tub2	1-a
(n=7)	2	2	por	0
	3	3	por	0
	4	3	por	0
	5	3	tub2	1-a
	6	3	muc	0
	7	3	tub2	1-a

a) According to the General Rules for the Gastric Cancer Study of the Japanese Research Society for Gastric Cancer.[15] *b*) Histological type: tub1, well differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; por, poorly differentiated adenocarcinoma; sig, signet cell carcinoma; muc, mucinous adenocarcinoma.

4.50±1.59 pmol/g in the AO-90 group and 4.98±2.50 pmol/g in the control group (no significant difference). The TS-total activity in cancer-free mucosa was 3.07± 1.55 pmol/g in the AO-90 group, and 4.23±1.70 pmol/g in the control group. There was also no significant difference between these values, or between the tumor tissue and cancer-free mucosal tissue in each group.

By contrast, the value of TS-free activity was 1.12± 0.74 pmol/g in tumors in the AO-90 group, being significantly lower than that in the control group, 2.35±0.74 pmol/g, at $P=0.0156$. The value of cancer-free mucosa was 1.15±0.52 pmol/g in the AO-90 group, lower than 1.85±0.93 pmol/g in the control group, though the difference was not significant.

The TSIR of tumor tissue was significantly higher in the AO-90 group (76.27±9.50%) than in the control group (45.87±26.43%) at $P=0.0243$. The TSIR of cancer-free gastric mucosa was 56.28±25.97% in the AO-90 group and 55.08±14.40% in the control group, being essentially the same.

Histopathological evaluation of the therapeutic response of gastric cancer Table V summarizes the results of histological evaluation of the anti-cancer effect in both groups.

In the AO-90 group, grade 2 was seen in 4 cases, grade 1-b in 1, grade 1-a in 1, and grade 0 in 1. In the control group, 3 cases were grade 1-a and the remaining 4 were grade 0. There was a statistically significant difference between the two groups in both degree and incidence of histological response at $P=0.016$ by the Mann-Whitney U test.

DISCUSSION

L-Methionine, a sulfur-containing amino acid, is essential for methylation, which occurs in the synthesis of DNA, RNA, proteins and other biochemicals. It was demonstrated that various malignant tumor cells were unable to proliferate in a medium deprived of L-methionine in tissue culture experiments.[17–20] The growth of Walker carcinosarcoma, implanted in rats, was suppressed by a transient decrease in the methionine level in the body.[19] We reported that it was possible safely to decrease the level of methionine in plasma, liver and tumor tissue *in vivo* by infusing an amino acid mixture lacking L-methionine and L-cysteine for total parenteral nutrition (Met-depleting TPN), resulting in some suppression of tumor growth in tumor-bearing animals.[5, 7, 21] It was also demonstrated that this parenteral treatment enhanced the effect of several anti-cancer agents.[3, 4, 6, 8–10] Furthermore, in clinical trials in gastrointestinal cancers including phase I, early phase II and late phase II, Met-depleting TPN combined with 5-FU administration showed a marked anti-tumor effect in advanced gastric cancer.[1, 2, 6, 11, 12] In particular, our recent multicenter late phase-II randomized trial in 138 advanced gastric cancers demonstrated that this parenteral treatment for 14 days with 5-FU and mitomycin C showed a higher therapeutic response (26.3%) than was obtained in the control group given conventional methionine-infusing TPN with 5-FU and mitomycin C (8.1%); the difference was significant.[12]

In this present study, preoperative Met-depleting TPN for only 8 days with a small dose of 5-FU (4.0 g/body in total) markedly degenerated the cancer tissue with a high frequency in the AO-90 group, as confirmed by detailed histopathological examination of the resected materials. It was reported that the histological response to chemotherapy or radiotherapy in gastrointestinal cancer tissues was best revealed two to three weeks after completion of the treatment.[22] In our experience, some patients with gastric cancer who received preoperative Met-depleting TPN treatment with 5-FU for more than 14 days showed a marked therapeutic response, such as complete diminution of the cancer, grade 3 response.[23] Therefore, the histological finding of enhanced anti-cancer effect under the conditions of this study is exciting.

In Sato lung carcinoma-bearing rats given Met-depleting TPN treatment with AO-90 as the only nitrogen source for 10 days, L-methionine concentration in the tumor tissue was significantly decreased by about 35% compared with that of the control group.[7] The same treatment enhanced the anti-cancer effect of 5-FU in

YS-bearing rats.[9] Methionine synthesis, namely methylation of homocysteine, involves two enzymes, MS1 and MS2.[24] Further, two mechanisms for the anti-cancer effect of 5-FU have been reported: one is the inhibition of DNA synthesis due to the limitation of TS activity[16] and the other is the reduction of RNA response.[25] Since an increase of CH_2FH_4 can reduce TS activity, we examined the changes in methionine synthase activity and the relation between the effect of 5-FU plus methionine depletion and folic acid metabolism in YS-bearing rats.[14] The level of MS1 in the Met-depleting TPN group was 2 times higher than in the control group infused with methionine, the tetrahydrofolate concentration was 3 times higher, and the CH_2FH_4 content was 2 times higher. TS-free activity in the YS tumor was reduced with a significant TSIR elevation, and YS tumor growth was inhibited by Met-depleting TPN and 5-FU in comparison with the control. In the present clinical study, the TS-free activity of tumorous tissue was also obviously reduced in human gastric cancer, although the number of cases was small, and there was a more significant elevation of TSIR than that in the YS-bearing rat experiment.

TS assays in this study were conducted according to Spears and Gustavsson[16] with a slight modification (Spears' method was developed for the hepatic tissue of rabbits). We found that the TS-free levels often exceeded TS-total when we assayed clinical samples, and the activity of TS-total decreased more sharply than that of TS-free. As we thought that TS-total activity would decrease during dissociation, we modified the conditions of the dissociation reaction as follows: 1) the reaction temperature was reduced from 30°C to 25°C; 2) the homogenizing buffer was changed from 0.2 *M* Tris buffer (pH 7.4) to 50 m*M* potassium phosphate buffer (pH 7.4); 3) the composition of dissociation buffer was modified from 0.2% gelatin(+) to 2% BSA(+). These changes prevented the decline of TS-total activity in comparison with that of TS-free (unpublished data). The TSIR values of cancer-free gastric mucosa in the AO-90 group and in the control group were found to be almost the same, suggesting that Met-depleting TPN may enhance the toxicity of 5-FU to the tumor cells more potently than to the non tumorous mucosal cells.

Because the amount of materials for analysis was limited in this study focusing on human gastric cancer, we could not assay MS1 activity, or tetrahydrofolate and CH_2FH_4 contents. But from the viewpoint of the changes in TS activity and TSIR in this study, we think that the same mechanisms as discussed above would have operated, resulting in the marked degeneration of the cancer tissue, despite the small dose of 5-FU and short (8-day) Met-depleting TPN treatment.

In conclusion, Met-depleting TPN may act as an effective biochemical modulator for 5-FU. This combined therapy seems to be a very effective treatment for gastric cancer without any notable adverse effects in a short period. However, many more patients shoud be assessed to confirm these findings, and fundamental studies are also necessary to clarify the synergistic effect of Met-depleting TPN with other anti-cancer agents including doxorubicin.[10]

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