ORIGINAL

Intraperitoneal infusion of paclitaxel with S-1 for peritoneal metastasis of advanced gastric cancer : phase I study

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Abstract : Background : Intraperitoneal administration of taxanes revealed excellent anti-tumor effect for peritoneal metastasis of gastric cancer in some experimental models. The aim of this study is to determine maximum tolerated dose (MTD), dose limiting toxicity (DLT) and recommended dose (RD) of intraperitoneally infused paclitaxel (PTX) with S-1 as a phase I study. Patients and Methods : Eighteen patients with advanced gastric cancer in addition to confirmed peritoneal metastasis using laparoscopy were enrolled in this study. The regimen consists of oral administration of S-1 (Dose 80 mg : BSA< 1.25 m^2, 100 mg : 1.25<BSA<1.5 m^2, 120 mg : BSA>1.5 m^2) for 14 days and intraperitoneal infusion of PTX (Dose escalation : level I : 40, II : 60, III : 80, level IV : 90, V : 100 mg/m^2) at day1 and 14. PTX concentrations in serum and ascites were determined at 4, 8, 12, 24, 48 hours after the infusion, which was repeated twice every 4 weeks. Results : The number of patients were as follows : Level I : 3, Level II : 6, Level III : 3, Level IV : 3, Level V : 3. Grade 3 leukocytopenia was confirmed in 1 (Level II) and 2 (Level V). MTD is 90 mg/m^2, RD is 80 mg/m^2 and DLT is Grade 3 leukocytopenia. The average serum PTX concentrations remained in optimal range except for all 3 of level V patients. In all cohorts, the PTX concentrations in the ascites were approximately 1000 folds higher than those in serum for 48 hours after the infusion. Conclusions : MTD and RD were PTX 90 mg/m^2, 80 mg/m^2, respectively. These findings were supported by pharmocokinetics of PTX J. Med. Invest. 58 : 134-139, February, 2011

Mini-Abstract : *In intraperitoneal infusion of PTX with S-1, DLT was leukocytopenia, MTD and RD were PTX 90 mg/m^2, 80 mg/m^2, respectively. These findings were supported by pharmocokinetics of PTX*

Keywords : *paclitaxel, S-1, intraperitoneal infusion, peritoneal metastasis, gastric cancer*

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INTRODUCTION

Median survival time, even with the best supportive care, for patients with unresectable or metastatic gastric cancer is only 3.1 months (1). Although peritoneum is the most common metastatic site of

advanced gastric cancer, a standard regimen has not been established despite the number of trials and the survival rate is very low.

Recently new chemotherapy agents have been developed. In particular S-1 revealed a high response rate of 49% for advanced gastric cancer in late phase II study (2), which has been widely accepted as a key drug even for adjuvant setting in Japan (3).

Taxanes stabilize and excessively form microtubules, which is a different mechanism from other agents. In phase II study, response rate of paclitaxel (PTX) for advanced gastric cancer was 21% and not affected by differentiation of adenocarcinoma (4, 5). High concentrations approximately 1000 times of PTX in the peritoneal cavity maintained compared with those in serum after intraperitoneal administration because of fat solubility and heavy molecular weight ; 853.92 (6). Excellent pharmacokinetics and anti-tumor effect to the peritoneal dissemination of gastric cancer was reported in the experimental model (7).

It is considered that S-1 and PTX is one of the best combinations for the treatment peritoneal metastasis of gastric cancer. The aim of this study is to determine the appropriate doses and feasibility of intraperitoneal infusion of paclitaxel (PTX) with orally administered S-1.

PATIENTS AND METHODS

Patient eligibility

Patients with peritoneal metastasis of advanced gastric cancer were eligible for this clinical trial. Before initiation of the study, relevant study documentation was submitted to and approved by the responsible ethics committee : the University of Tokushima hospital clinical research Ethical Review Board, Tokushima, Japan.

The guidelines of the World Medical Association Declaration of Helsinki in its revised edition (Edinburgh, Scotland, October 2000) and other applicable regulatory requirements were strictly followed. Written informed consent was obtained from each patient before any study-specific screening procedures were undertaken.

Inclusion criteria

Patients aged 20-75 years, had to have histologically or cytologically confirmed peritoneal metastasis of gastric cancer using laparoscopy under general anesthesia, who had not received abdominal surgery and any prior chemotherapy regimens.

Exclusion criteria

Patients with ischemic heart disease that needed medication, liver cirrhosis, lung fibrosis, pneumonia, intestinal bleeding or other severe complications were excluded.

Treatment plan

An initial laparoscopy was performed under general anesthesia for the patients with advanced gastric cancer histologically diagnosed. Peritoneal metastasis was histologically confirmed by removal of disseminated nodules or peritoneal cytology.

The catheter for intraperitoneal infusion of PTX was passed through the wound of trocar port in the right side of the umbilicus, which was connected to the port implanted in the abdominal wall for the patient diagnosed peritoneal metastasis.

S-1 was orally administered with a fixed quantity (Dose 80 mg : Body Surface Area (BSA) < 1.25 m^2, 100 mg : 1.25 < BSA < 1.5 m^2, 120 mg : BSA > 1.5 m^2) for 14 days. PTX was infused intraperitoneally through the implanted catheter at day1 and 14. Dose of PTX was escalated ; level I : 40 mg/m^2, level II : 60 mg/m^2, level III : 80 mg/m^2 level IV : 90 mg/m^2, level V : 100 mg/m^2. Intraperitoneal PTX with S-1 was repeated two cycles every four weeks.

Adverse events were coded according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE), version 3.0. Dose Limiting Toxicity (DLT) was defined two patients had nonhematologic or hematologic grade 3 or greater adverse events. If one patient had Grade 3 or more adverse events, the cohort was expanded to three patients owing to occurrence of a DLT. As a result, the dose of PTX was increased to the level that two patients had a DLT in turn. The Maximum Tolerated Dose (MTD) was defined as one escalation level lower than that DLT was confirmed. Recommended dose (RD) was defined as one level lower than MTD.

Analytic methods and pharmocokinetics

Blood samples for pharmacokinetic analysis were drawn before infusion, at 4, 8, 12, 24 and 48 h after the infusion of PTX. Ascites samples were aspirated through the catheter for PTX infusion at the same time points. High performance liquid chromatography (Ultra-Violet absorbance detector : Ultra-violet of 227 nm in wave length) was used to analyze PTX concentrations of serum and ascites in SRL, Inc

(Tokyo, Japan).

RESULTS

Patient demographics

Patient demographics are shown in Table 1. The 18 patients were enrolled in this study after histologically confirming peritoneal metastasis. Two of the 18 patients had adenocarcninoma cells in peritoneal cytology without macroscopically detected metastatic nodules. Curative operation was not impossible for all 18 patients.

Table 1 : Patient demographics

Sex (male/female)	14/4
Age (years) (median/min/max)	56/49/75
WHO Performance status (0/1)	14/4
Macroscopic types III / IV	10/8
Histological typing well/poorly differentiated	3/15
Positive adenocarcinoma cells in peritoneal cytology	18
Macroscopically detected metastatic nodules	16
Gastrectomy	12

Clinical safety and tolerability

All 18 enrolled patients were evaluated for safety. A summary of the patient- and investigator-reported drug related clinical adverse events is shown in Table 2. Current regimen was generally well tolerated, with 6 patients clinically significant drug-related adverse events. The most frequently reported adverse event was Grade 3 leukocytopenia. Grade 1 or 2 anemia, vomiting and abdominal pain were confirmed.

The 40, 80, 90 and 100 mg/m^2 cohort enrolled three patients. After the one patient had Grade 3 leukocytopenia in 60 mg/m^2 cohort, this cohort was expanded to 6 patients without Grade 3 or more adverse events. Grade 3 leukocytopenia was confirmed consecutively 2 patients in 100 mg/m^2 cohort.

DLT was leukocytopenia, MTD was 90 mg/m^2 and RD was 80 mg/m^2, respectively.

Table 2 : Drug-related adverse events

	Grade 1	Grade 2	Grade 3	Grade 4
BLOOD/BONE MARROW				
Hemoglobin	1 (5.6%)			
Leukocytes			3 (16.7%)	
GASTROINTESTINAL				
Vomiting	1(5.6%)			
PAIN				
Abdominal pain		1 (5.6%)		

Pharmocokinetics of PTX

The average serum PTX concentrations in 40, 60, 80 and 90 mg/m^2 cohort were maintained between the lower limit of cytotoxic effects and upper limit of blood system disorder, which were over upper limit of blood system disorder in all 3 patients of 100 mg/m^2 cohort. In all cohorts, PTX concentrations in the ascites were approximately 1000 folds higher than those in serum for 48 hours after the infusion (Figure 1).

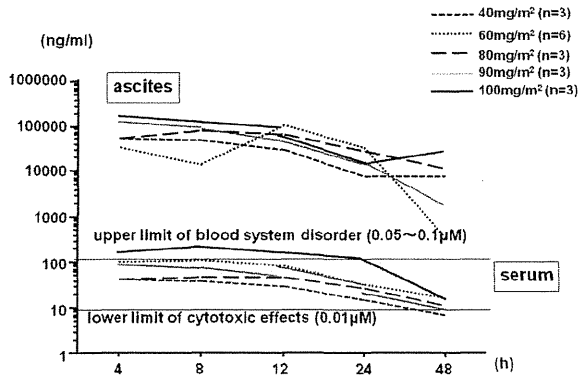

Figure 1 : Pharmacokinetics of PTX
The PTX concentrations in 40, 60, 80 and 90 mg/m^2 cohort remained in the optimal range. In 100 mg/m^2 cohort, the PTX concentrations were over upper limit of blood system. PTX concentrations in the ascites were approximately 1000 times higher than those in serum.

Clinical activity

All 18 patients were evaluated for efficacy. Objective clinical response was assessed according to Response Evaluation Criteria in Solid Tumors (RECIST). The 2 patients had partial response. The 15 patients was recorded as stable disease, however, positive adenocarcinoma cells in peritoneal cytology became negative in 2 patients, remarkable decrease of ascites was found in 2 patients. Down staging according to the 13th Japanese Classification of Gastric Carcinoma was possible in 2 patients (2 : positive cytology became negative). There was one patient classified as having progressive disease.

Gastrectomy was performed for 12 of 18 patients, which had curative potential in the patients with down staging. The median survival time was 11 months. Survival time of the 2 patients whose positive cytology became negative was 32 and 48 months, respectively (Table 3).

Table 3 : Clinical activity

Case	Level	RECIST	Down staging	Gastrectomy	Prognosis	Survival time (months)
1	1	PR	-	+	death	8
2	1	SD	-	+	death	10
3	1	SD	+*	+	alive	48
4	2	SD	-	+	death	17
5	2	PR	-	+	death	21
6	2	SD	-	-	death	15
7	2	SD	-	-	death	14
8	2	SD	-	+	death	10
9	2	SD	-	-	death	11
10	3	SD	+*	+	alive	32
11	3	SD	-	+	alive	30
12	3	SD	-	+	death	8
13	4	SD	-	+	death	9
14	4	SD	-	-	death	6
15	4	SD	-	+	death	5
16	5	PD	-	-	death	14
17	5	SD	-	-	death	7
18	5	SD	-	+	alive	11

* Positive adenocarcinoma cells in peritoneal cytology became negative.

DISCUSSION

Intraperitoneal infusion of PTX was generally well tolerated. The most frequently reported adverse event was Grade 3 leukocytopenia. DLT was leukocytopenia, MTD was 90 mg/m^2 and RD was 80 mg/m^2, respectively. These findings were supported by pharmacokinetics of PTX.

Because S-1 is the most widely accepted drug for gastric cancer in Japan, a lot of combination trials based on S-1 have been performed (8-10). Median overall survival was significantly longer in patients assigned to S-1 plus cisplatin (13.0 months) than in those assigned to S-1 alone (11.0 months) in the Phase III trial for advanced gastric cancer, however, peritoneal dissemination held 34%, 24% of each group, respectively (8). Significant differences in overall survival compared with S-1 alone revealed in any other regimens. It has not been established standard regimens for peritoneal metastasis of gastric cancer.

Intraperitoneal PTX in the phase II trial for the patients with small-volume residual carcinomas of the ovary, fallopian tube, or peritoneum was well tolerated, which included only moderate abdominal pain (grade 2 : 15.7%, grade 3 ; 1.3%) and minimal neutropenia (grade 2 ; 3.9% ; grade 3 ; 1.3%) (11). The incidence of Grade 3 neutropenia were observed in 32% of the patients with advanced gastric cancer in the treatment schedule comprised an intravenous infusion of 80 mg/m^2 PTX , repeated weekly three times for 4 weeks (12). These data suggested that intraperitoneal administration of PTX did not increase the incidence of drug-induced toxicities (13).

A pharmacokinetics study demonstrated that the PTX concentration in ascites remained in the range of the lower limit of cytotoxic effects and upper limit of blood system disorder from 4 to 72 hours after intravenous infusion of 60 and 80 mg/m^2 PTX. On the other hand, plasma concentrations of PTX were over upper limit of blood system disorder at 4 hours (14). In contrast, the PTX concentrations in 40, 60, 80 and 90 mg/m^2 cohort in this study remained in the optimal range. In 100 mg/m^2 cohort, the PTX concentrations were over upper limit of blood system. PTX concentrations in the ascites were approximately 1,000 folds higher than those in serum.

A major advantage after intraperitoneal delivery of PTX is high concentration in the peritoneal cavity (550-2,000 folds) compared with the systemic compartment (13). Drug exposure of high concentration is considered to have an advantage because antitumor effects increased dose dependent manner

as far as could be seen there were no severe toxicities in the experimental model (7).

Although this study is a phase I study, the response rate and survival could not be described exactly, two patients with positive adnocarcinoma cells and no macroscopically detected disseminate nodules had a long survival of over 30 months. The overall 5-year survival (43.8%) of advanced gastric cancer patients with intraperitoneal free cancer cells without overt peritoneal metastasis (CY+/P-) after extensive intraoperative peritoneal lavage followed by the intraperitoneal chemotherapy (EIPL-IPC: peritoneal lavage of 10 times using 1 L of physiological saline following cisplatin at a dose of 100 mg/body into the peritoneal cavity) was significantly better than that of the intraperitoneal chemotherapy (4.6%) and the surgery alone (0%) (15). It is important to detect positive adenocarcinoma cells in the peritoneal cavity to improve survival of the patients with peritoneal metastasis (16).

Concerning patients with macroscopically detected peritoneal metastasis, the utility of peritonectomy with chemohyperthermic peritoneal perfusion (CHPP) was reported, however, there are some problems regarding peritonectomy : complicated procedures and CHPP : severe stress to the patients and needs of specific and expensive instruments (17).

Fat solubility of PTX is suitable for intraperitoneal infusion, in contrast, Cremophor EL and ethanol is necessary as a solvent for clinical use, which causes acute hypersensitivity (18). For better and safe drug delivery system, various modifications of PTX have been developed and phase I trials were reported (19-21). Intraperitoneal PTX using the water-soluble solvent revealed excellent pharmacokinetics compared with Cremophor EL (22).

Intraperitoneal PTX including new modified drugs has high potentials to improve survival for the peritoneal metastasis of gastric cancer.

CONFLICT OF INTEREST STATEMENT

Mitsuo Shimada received a research grant from Research Support Foundation of the University of Tokushima and TAIHO Pharmaceutical. Co. Ltd. ; Other authors have no conflict of interest.

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ORIGINAL

Effect of histone deacetylase inhibitor in combination with 5-fluorouracil on pancreas cancer and cholangiocarcinoma cell lines

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Abstract : Background : Histone deacetylase (HDAC) is well known to be associated with tumorigenesis through epigenetic regulation, and its inhibitors (HDACIs) induce differentiation and apoptosis of tumor cells. We examined the therapeutic effects of valproic acid (VPA, a HDACI) with a combination of 5-fluorouracil (5-FU) in vitro. Methods : A human pancreas cancer cell line (SUIT-2) and a cholangiocarcinoma cell line (HuCCT1) were used. Cell viabilities were evaluated by a cell proliferation assay. We determined the anticancer effects of VPA combined with 5-FU in these cell lines. Results : Pancreas cancer (SUIT-2) : No effect of 5-FU (1.0 μM) was observed, but 17% and 30% of proliferation-inhibitory effects were recognized in a dose of 2.5 or 5.0 μM, respectively. Cell viability was only weakly reduced by VPA (0.5 mM). However, in combination of 5-FU (1.0 μM) with VPA (0.5 mM), 19% of inhibitory effect was observed. Cholangiocarcinoma (HuCCT1) : 5-FU (1.0 μM) did not suppress the cell viability, but 5-FU (2.5 μM) suppressed by 23%. VPA (0.5 mM) did not suppress the cell viability, while VPA (1.0 mM) weakly decreased it by 11%. Combination of 5-FU (1.0 μM) and VPA (0.5 mM) markedly reduced the cell viability by 30%. Conclusion : VPA augmented the anti-tumor effects of 5-FU in cancer cell lines. Therefore, a combination therapy of 5-FU plus VPA may be a promising therapeutic option for patients with pancreas cancer and cholangiocarcinoma. J. Med. Invest. 58 : 106-109, February, 2011

Keywords **:** *pancreas cancer, cholangiocarcinoma, HDAC inhibitor, valproic acid, epigenetic regulation*

INTRODUCTION

Pancreas cancer is one of the most aggressive human cancers. The overall 5-year survival rate among patients with pancreatic cancer is < 5% (1). Cholangiocarcinoma is a cancer arising from bile duct epithelium. This cancer is one of the most difficult diseases to treat as pancreas cancer, and no standard chemotherapy has been established (2, 3). Therefore, we have researched about resistance of chemotherapy in pancreatic and biliary tract cancers.

5-fluorouracil (5-FU) is a chemotherapeutic drug which is widely used mainly for the treatment of the digestive system cancer, but the response rate

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in pancreatic and biliary tract cancers is very low (4, 5). Therefore, new agents and innovative approach to therapy are the important subjects for research.

Alterations in the epigenetic modulation of gene expression have been implicated in cancer development and progression, and histone acetylation, one of the epigenetic regulations, is a posttranslational modulation of the nucleosomal histones that affects chromatin structure and modulates gene expressions. Histone deacetylases (HDACs) comprise an ancient family of enzymes that play crucial roles in numerous biological processes (6), and HDACs are found to be overexpressed in many tumor types (7, 8). We reported that the survival rate for pancreas cancer patients with HDAC1-positive was significantly lower than that for patients with HDAC1-negative, and HDAC1 was considered to be a promising therapeutic target in pancreas cancer (9). HDAC inhibitors induce the differentiation or apoptosis of cancer cells (10, 11). Therefore, HDAC inhibitors are promising new agents, in this study, we used Valproic acid (VPA). VPA has the antitumor effects of a HDAC inhibitor (12), and VPA has been shown to have anticancer effects in various cancer models (13).

The aim of this study was to investigate the anticancer effects of VPA in combination with 5-FU in pancreas cancer and cholangiocarcinoma cell lines.

MATERIAL AND METHOD

Cell lines and culture conditions

SUIT-2 cell was purchased from the Japanese Collection Research Bioresources Cell Bank (Tokyo, Japan). HuCCT-1 was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. All cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 70 μg/mL penicillin and 100 μg/mL streptomycin (complete medium) and maintained at 37°C in a humidiWed incubator with 5% CO_2 in air. The cells were maintained for no longer than 12 weeks after recovery from frozen stock.

Reagents

Valproic acid was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and kept at 4°C and diluted in PBS as necessary at the time of use. 5-FU was purchased from Kyowa Hakko (Tokyo, Japan) and made fresh in 0.9% NaCl on the day of use.

Cell proliferation assay

All of tumor cells (5×10^3) were seeded into 38-mm^2 wells of flat-bottomed 96-well plates in quadruplicate and allowed to adhere overnight. The spent medium was then removed, and the cultures were refed with new medium (negative control) or medium containing diVerent concentrations of VPA and 5-FU. Incubation was continued for 72 h prior to adding the Cell Counting Kit-8, and after 2 h, the optical density was measured at 450 nm with a microplate reader (Multiskan JX ; Labsystems).

Statistical analyses

Statistical comparisons of mean values were conducted using oneway ANOVA. All the results are presented as mean ± SD. Statistical analysis was performed using Stat View 5.0 J software (SAS Institute, Inc., Cary, NC, USA). A P value of less than 0.05 was considered to be statistically significant.

RESULTS

In pancreas cancer cell line, SUIT-2, no effect of 5-FU was observed in dose of 1.0 μM and 17%, 30% and 33% of proliferation-inhibitory effects were observed in dose of 2.5, 5.0 and 10 μM (Fig. 1A). VPA (0.5 mM) weakly decreased cell viability by 13%, and VPA (1.0 mM) suppressed by 19% (Fig. 1B). In combination of 5-FU and VPA, 19% of inhibitory effect was observed in dose of 5-FU 1.0 μM/VPA 0.5 mM, the combination effect was significant compare

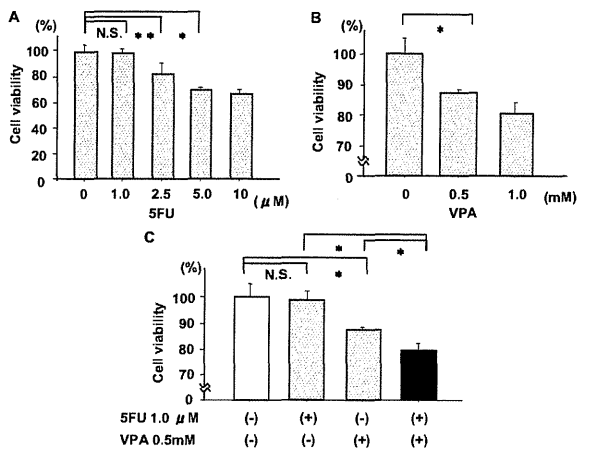

Figure 1 : The effect of 5-FU (A), VPA (B) and combination of 5-FU and VPA (C) in inhibiting cell proliferation of human pancreas cancer cell line, SUIT-2.
** : $p<0.05$, * : $p<0.01$.

to 5-FU alone or VPA alone ($P<0.01$) (Fig. 1C).

In cholangiocarcinoma cell line, 5-FU (1.0 μM) did not suppress the cell viability, 5-FU (2.5 μM) suppressed by 23%, and 34% and 39% of proliferation-inhibitory effects were observed in dose of 5.0 and 10 μM (Fig. 2A). VPA (0.5 mM) did not suppress the cell viability, while VPA (1.0 mM) weakly decreased it by 11% (Fig. 2B). 5-FU (1.0 μM) and VPA (0.5 mM) reduced by 30%, which significantly augmented the anticancer effect of 5-FU alone or VPA alone ($P<0.01$) (Fig. 2C).

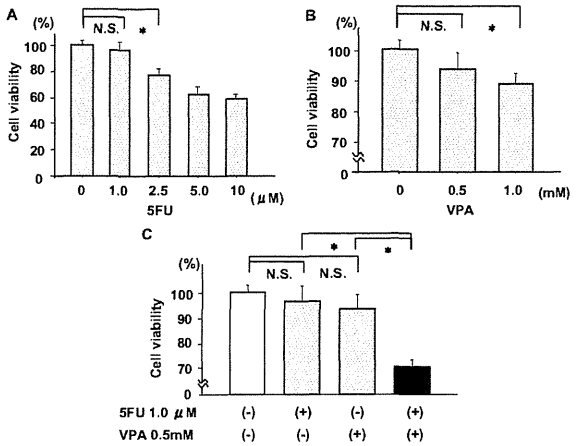

Figure 2 : The effect of 5-FU (A), VPA (B) and combination of 5-FU and VPA (C) in inhibiting cell proliferation of human cholangiocarcinoma cell line, HuCCT1.
* : $p<0.01$.

DISCUSSION

In the present study, we assessed the effect of HDAC inhibitor (VPA) in combination with 5-FU on pancreatic-biliary carcinoma cell lines. To our knowledge, this is the first report to show that VPA enhances the effect of 5-FU on both pancreas cancer and cholangiocarcinoma cell lines.

HDAC inhibitors are useful in cancer treatment when used in combination with current chemotherapeutic drugs, especially in combination with 5-FU, HDAC inhibitor (MS275) enhance the effect of 5-FU in colorectal cancer cells (14), and other HDAC inhibitor (SAHA) enhance the effect of 5-FU in non-small cell lung cancer (15). The mechanisms of the additional effects on HDAC inhibitors to the cytotoxic agent are the enhancement of apoptosis (14) and the up-regulation of p21 (waf1/cip1) expression (15). In this study, the mechanisms may be the augmentation of apoptosis or the enhancement of p21 (waf1/cip1) expression.

However, some HDAC inhibitors are of limited therapeutic use due to toxic side effects at high doses (16). VPA is widely used as a therapeutic drug for epilepsy, its toxicity profile and pharmacokinetic properties are well established. Furthermore, in our study, the dose of VPA was 0.5 mM, because the peak plasma concentration in patients treated for epilepsy ranges between 0.5 and 1.2 mM (17). VPA at a dose of 0.5 mM may not cause any serious side effects in clinical setting.

Recently, S-1, an oral drug consisting of the 5-FU prodrug tegafur, combined with two modulators of 5-FU activity, has been developed (18-20). S-1 contains 5-chloro-2,4-dihydroxypyridine (CDHP), CDHP competitively inhibits the 5-FU degradative enzyme dihydropyrimidine dehydrogenase (DPD), resulting in the retention of a prolonged concentration of 5-FU in blood (18).

VPA has been investigated in clinical studies (21, 22), we plan the clinical trial of the combination therapy, S-1 and VPA. We have expected VPA enhances the anti-tumor effect of S-1 in this trial.

In conclusion, VPA augmented the inhibitory effects of 5-FU on the proliferation rates of both pancreas cancer and cholangiocarcinoma cell lines. Therefore, VPA in combination with 5-FU is suggested to be a promising therapeutic option for pancreatic and biliary tract cancers.

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Original Article

Role of dihydropyrimidine dehydrogenase and thymidylate synthase expression in immunohistochemistry of intrahepatic cholangiocarcinoma

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Aims: Dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TS) are key enzymes in the metabolism of 5-fluorouracil and have been implicated as possible prognostic markers for cancer patients. However, the clinical roles of DPD and TS in intrahepatic cholangiocarcinoma (IHCC) have not been investigated. The aim of this study was to clarify the clinicopathological role of DPD and TS expressions in IHCC.

Methods: Twenty-nine patients who had undergone hepatic resection for IHCC were enrolled in this study. Expressions of DPD and TS in the resected IHCC specimens were examined using anti-DPD or anti-TS antibody. The patients were divided into positive and negative groups according to DPD/TS expressions: DPD-positive group ($n = 18$) and DPD-negative group ($n = 11$)/TS-positive group ($n = 14$) and TS-negative group ($n = 15$). Clinicopathological factors were compared between the two groups.

Results: The overall survival rate was significantly lower in the DPD-negative group than in the DPD-positive group (1-year 36.4% vs. 77.4%, 3-year 18.2% vs. 43.0%; $P < 0.05$). The disease-free survival rate in the DPD-negative group tended to be lower than that in the DPD-positive group. The overall survival rate or disease-free survival rate did not appear to be associated with the TS-expression status. The Ki-67 labeling index in the DPD-negative group was significantly higher than that in the DPD-positive group ($16.9 \pm 3.2\%$ vs. $13.2 \pm 3.3\%$; $P < 0.05$).

Conclusions: The negative DPD expression was significantly associated with the enhanced tumor cell proliferation and poorer prognosis in patients with IHCC. DPD expression is a potential prognostic indicator for IHCC.

Key words: intrahepatic cholangiocarcinoma, Ki-67 index, prognosis, recurrent pattern

INTRODUCTION

INTRAHEPATIC CHOLANGIOCARCINOMA (IHCC) accounts for five percent of primary malignant liver tumors, arising from biliary epithelium,[1] and well known to be one of the most malignant solid tumors found in the digestive organs.[1–5] This highly malignant carcinoma is associated with lymph node metastasis, intrahepatic metastasis, peritoneal dissemination, bile duct invasion, and vascular invasion.[2–5] Prognosis of IHCC is very poor with a 5-year survival rate ranging from 25% to 35%.[1–5] Therefore, it is important to elucidate tumor characteristics and prognostic factors after surgical resection for IHCC.

Dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TS) act as key enzymes of pyrimidine cascade and 5-fluorouracil (5-FU) metabolism.[6–8] In this cascade, 5-FU is first catabolised by DPD. TS decreases the synthesis of deoxythymine monophosphate from deoxyuridine monophosphate, and exhibits antitumor effects.[8]

Expressions of DPD and TS are correlated with the antitumor effects of 5-FU and 5-FU based chemotherapy, such as tegafur-uracil, and S-1.[9–11] Recently, DPD and TS have been reported to play an important role in various kinds of cancers. Down regulation of DPD gene expressions may enhance the negative prognostic effect in colorectal tumors[12] and ovarian cancer.[13]

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Overall survival was significantly better in the TS negative patients than in the TS positive patients among resected colon cancer patients.[14] High expression of TS in tumors enhanced distant metastasis after surgery.[15]

We have previously reported that a low expression of DPD mRNA was a poor prognostic factor in hepatocellular carcinoma (HCC).[16] However, to the best of our knowledge, only one *in vitro* study examined the DPD and TS expressions in IHCC cell lines.[17]

This is the first report evaluating the association of the DPD and TS expressions with the clinicopathological variables in surgical patients with IHCC.

PATIENTS AND METHODS

Patients

TWENTY-NINE PATIENTS who had undergone surgical resection for IHCC at Tokushima University Hospital between 1992 and 2009 were included in this study. There were 20 men and 9 women, with a mean age of 66.9 years (range, 43–84 years). In 19 patients (65.5%), hepatic lobectomy was performed. Lymph node dissections of the hepatoduodenal ligament and along the common hepatic artery or more extended lymphadenectomies were performed in 14 patients (48.3%). Extrahepatic bile duct resections were performed on 11 patients (37.9%). Consequently, 22 patients (75.9%) had received R0 or R1 resections. None of the patients received prior chemotherapy or irradiation before surgical resection. Mean follow-up period was 29 months (range, 2–111 months). The clinical stages were defined according to the Classification of Primary Liver Cancer Study Group of Japan.[18]

The current study was authorized in advance by the Institutional Review Board of the University of Tokushima, and all patients provided written informed consent.

Immunohistochemistry

The expressions of DPD and TS in the resected IHCC specimens were evaluated with using immunohistochemistry as described previously.[19,20] Surgical specimens were fixed in 10% formaldehyde embedded in paraffin and cut into 4-μm thick sections. Sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Deparaffinized sections were retrieved by microwaving for 20 min. Endogenous peroxidase activity was blocked by soaking the sections in 0.3% hydrogen peroxide in methanol for 30 min. After washing with PBS, sections were placed in normal goat serum (2% in PBS) for 30 min to reduce nonspecific staining. The sections were subsequently incubated with DPD antibody (rabbit polyclonal, dilution 1:200; Taiho Pharmaceutical, Tokushima) or TS antibody (rabbit polyclonal, dilution 1:200; Taiho Pharmaceutical), overnight at 4 °C in moist chambers. The sections were incubated with goat anti-mouse immunoglobulin for 20 min and then with horseradish peroxidase-conjugated streptavidin complex (Histofine SAB-PO Kit; Biogenex Laboratories, Tokyo). To visualize immunoreactivity, diaminobenzidine/H_2O_2 (1 mg/mL) in PBS was used as the substrate. The sections were counter stained with hematoxyline, dehydrated with ethanol, and treated with xylene.

Assessment of DPD and TS staining was expressed as the percentage of stained cells in the cytoplasms out of total number of tumor cells and devided into two groups as follows: <5%; negative expression, ≥5%; positive expression (Fig. 1).[19] The assessment of immunohistochemistry was conducted without knowledge of the results of other experiments.

Determination of the Ki-67 labeling index

The correlation between the Ki-67 labeling index and DPD or TS expression was investigated. Determination of the Ki-67 labeling index was previously reported.[16] Five hundred tumor cells were counted in each 4-μm thick section. The Ki-67 labeling index was defined as the number of Ki-67 positive nuclei divided by total number of cancer cells, and expressed as a percentage.

Statistics

All statistical analysis was calculated through Stat View statistical software (Stat View 5.0; SAS Institute, Cary, NC). Relationships between DPD or TS expression and the clinicopathological variables were analyzed with the χ^2 test and Mann–Whitney *U*-test. Survival curves were calculated using the Kaplan–Meier method and compared using the Wilcoxon test. All significant factors by univariate analysis were included in the Cox's proportional hazards model of multivariate analysis to identify independent factors influencing survival. Statistical significance was defined as $P < 0.05$.

RESULTS

Immunohistochemistry

IN DPD EXPRESSION, there were 18 (62.1%) positive and 11 (37.9%) negative cases. Regarding TS expression, there were 14 (48.3%) positive and 15 (51.7%) negative cases.

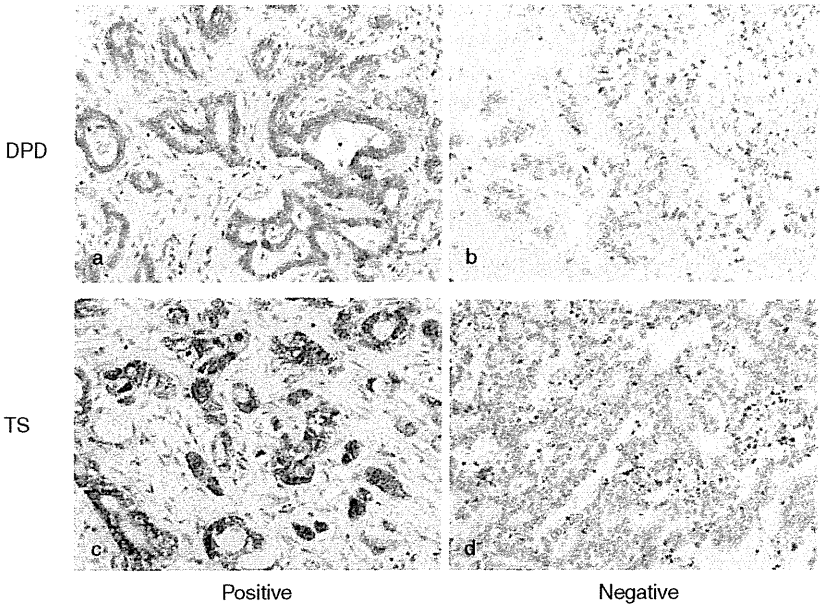

Figure 1 Dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TS) expressions in intrahepatic cholangiocarcinoma (IHCC). The positive immunostaining of DPD or TS was recognized in cytoplasm of cancer cells (a) DPD-positive (b) DPD-negative (c) TS-positive (d) TS-negative.

Correlation between DPD/TS expressions and clinicopathological characteristics

No significant correlations were observed in any clinicopathological variables, such as staging, curability, vascular invasion, intrahepatic metastasis, and other tumor factors according to the expression levels of DPD or TS. However, in the DPD negative group, the tumor tended to be located more frequently in the hilar region (Table 1). In the TS positive group, the incidences of advanced clinical stage, non curative surgical resection, larger tumor size, vessels infiltration, and intrahepatic metastasis tended to be higher than in the TS negative group (Table 2).

Overall and disease-free survival according to DPD/TS expressions

Figure 2 shows overall and disease-free survival rates according to DPD/TS expressions. The overall survival rate was significantly lower in the DPD-negative group than in the DPD positive group (1-year 36.4% vs. 77.4%, 3-year 18.2% vs. 43.0%; $P < 0.05$) (Fig. 2A). However, there were no differences in the overall survival rate between the TS-negative and the TS-positive group (1-year 58.2% vs. 64.3%, 3-year 39.9% vs. 26.8%; Fig. 2B).

Similarly, the disease-free survival rate in the DPD-negative group tended to be lower than in the DPD-positive group (1-year 22.2% vs. 57.1%, 3-year 11.1% vs. 31.2%), although there was no statistical significance

Table 1 Clinicopathological characteristics according to dihydropyrimidine dehydrogenase (DPD) expression

Factors	DPD expression		*P*-value
	Positive ($n = 18$)	Negative ($n = 11$)	
Mean age (years)	68.3 ± 7.5	64.5 ± 13.7	0.448
Sex (Male/Female)	13/5	7/4	0.628
Virus ([-]/HBV/HCV/Combined)	9/4/4/1	10/1/0/0	0.140
Staging (I, II/III, IV)	5/13	3/8	0.976
Curability (R0, 1/2)	14/4	8/3	0.758
Location (hilar/peripheral)	4/14	5/6	0.190
Tumor diameter (<4 cm/≥4 cm)	9/9	5/6	0.812
Macroscopic type: T/T + I	9/9	4/7	0.474
Differentiation: Diff./Undiff.	7/11	4/7	0.892
LN metastasis: −/+	6/12	4/7	0.868
Vessels infiltration: −/+	7/11	6/5	0.412
Intrahepatic metastasis: −/+	12/6	9/2	0.376

Diff, differentiation; T, mass-forming type; T + I, mass-forming + periductal infiltrative type; Undiff, undifferentiation.

Table 2 Clinicopathological characteristics according to TS expression

Factors	TS expression		*P*-value
	Positive ($n = 14$)	Negative ($n = 15$)	
Age: Mean	68.3 ± 7.5	64.5 ± 13.7	0.448
Gender: Male/Female	10/4	10/5	0.782
Virus:(–)/HBV/HCV/ Combined	9/2/3/0	10/3/1/1	0.528
Staging: I, II/III, IV	2/12	6/9	0.122
Curability: R0, 1/2	9/5	13/2	0.159
Location: Hilar/ Peripheral	4/10	5/10	0.782
Tumor diameter: <4 cm/ ≥4 cm	5/9	9/6	0.191
Macroscopic type: T/T + I	5/9	8/7	0.340
Differentiation: Diff./ Undiff.	4/10	7/8	0.316
LN metastasis: –/+	9/5	10/5	0.893
Vessels infiltration: –/+	4/10	9/6	0.089
Intrahepatic metastasis: –/+	8/6	13/2	0.076

Diff, differentiation; T, mass-forming type; T + I, mass-forming + periductal infiltrative type; Undiff, undifferentiation.

(Fig. 2C). No significant difference in the disease-free survival rate was observed between the TS-negative and the TS-positive group (1-year 46.2% vs. 40.0%, 3-year 23.1% vs. 20.0%; Fig. 2D).

Univariate and multivariate analysis of prognostic factors

Table 3 shows the results of univariate and multivariate analysis of prognostic factors. Univariate analysis revealed that location, differentiation, macroscopic type, vessels infiltration and intrahepatic metastasis were not significant factors in terms of postoperative survival. In contrast, curability ($P = 0.0009$), tumor size ($P = 0.0066$), lymph nodes metastases ($P = 0.0127$), and negative expression of DPD ($P = 0.0498$) were found to be significant prognostic factors for survival after surgical resection. In multivariate analysis using the Cox's proportional hazard model, tumor size (≥4 cm) was found to be an only independent prognostic factor. Negative expression of DPD tended to be an independent prognostic factor, although there was no statistical significance ($P = 0.1293$).

Recurrent pattern

Table 4 shows the correlation between the recurrent patterns and the DPD expression status. The recurrence rate in the DPD-positive group was similar to that in the DPD-negative group. However, in the DPD-negative group, the incidence of recurrence in the liver was significantly higher ($P < 0.05$) and that of lymph node and remote organ tended to be higher compared to the DPD-positive group.

Ki-67 proliferating index

The Ki-67 labeling index in the DPD-negative group was significantly higher than the DPD-positive group (16.9 ± 3.2% vs.13.2 ± 3.3%) ($P < 0.05$). There was no difference in the Ki-67 labeling index between the TS-positive group and the TS-negative group (14.1 ± 3.8% vs.15.5 ± 3.5%).

DISCUSSION

THESE RESULTS SHOW that negative DPD expression was significantly associated with poorer prognosis, higher proliferation index, and a higher incidence of recurrence in the liver. However, TS expression was not related to patient prognosis after surgical resection.

Several other reports have documented that the expression of DPD is related to prognostic and clinicopathological factors.[12,13] In primary liver cancer, however, only a few reports are available on the role of DPD.[21,22] The DPD activity in HCC was lower than in non cancerous tissue and a gradual decrease in DPD activity was associated with liver damage.[21] We previously reported that low mRNA expression of DPD was a poor prognostic factor and significantly related to advanced clinical stage, undifferentiated histology, microscopic intrahepatic metastasis, and related to tumor proliferation in HCC.[16] Regarding biliary tract cancer, DPD concentration was higher in cancerous tissue than in noncancerous tissue, although the prognosis is not different.[23] Ajiki *et al.* reported DPD expression was not a prognostic factor in gallbladder cancer.[24]

In this study, the patients in DPD-negative group had a significantly poorer prognosis. The mechanism of such results is not fully understood at present. However, in pyrimidine cascade, negative expression of DPD leads the synthesis of both uracil and thymine. Increased synthesis of uracil and thymine may relate to the enhancement of pyrimidine nucleotide pools[25] and may cause the cell proliferation of IHCC. Johnston SJ *et al.* suggested that the down-regulation of DPD expression might create a favorable environment for tumor growth.[26] Further, it was suggested that low DPD expression was associated with an increase in the metastatic activity. DPD activity in highly malignant murine neu-

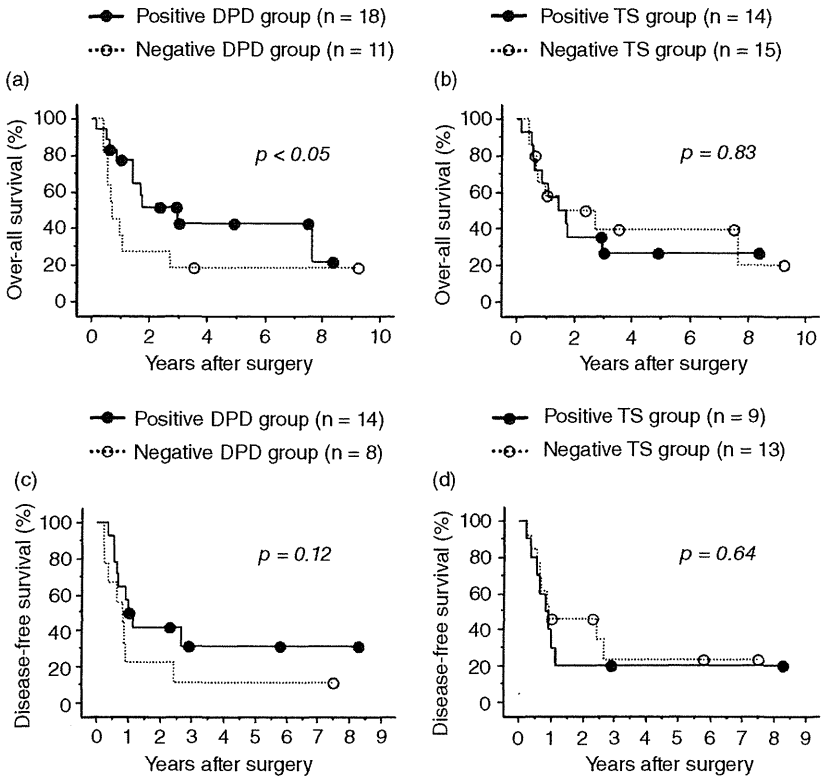

Figure 2 Overall survival rates in positive and negative expression of dihydropyrimidine dehydrogenase (DPD; a) and thymidylate synthase (TS; b). The survival rate in DPD-negative group was significantly lower than in the DPD-positive group. Disease-free survival rates in positive and negative expression of (c) DPD and (d) TS.

roblastoma cell line was lower than that in low malignant cell line.[27,28] Consistent with these reports, DPD-negative group had a significantly higher Ki-67 labeling index and higher incidence of recurrence in the liver than the DPD-positive group in our study.

Table 3 Univariate and Multivariate analysis of prognostic factors

	DP D positive ($n = 14$)	DPD negative ($n = 8$)	*P*-value
Recurrence	9	6	0.604
Recurrent pattern			
Liver ($n = 12$)	9	3	0.018
Lymph nodes ($n = 4$)	1	3	0.095
Peritoneum ($n = 1$)	0	1	0.205
Remote organ ($n = 2$)	0	2	0.063

Table 4 Correlation between DPD expression and recurrent site in patients with R0/1 resection ($n = 22$)

	Univariate *P*-value	Hazard ratio	Multivariate 95% C.I.	*P*-value
Curability: R2	0.0009	2.832	0.804–10.000	0.1052
Tumor Diameter: ≥4 cm	0.0066	4.413	1.427–7.705	0.0099
Lymph nodes metastasis: +	0.0127	2.921	0.851–10.030	0.0885
DPD expression: –	0.0498	2.132	0.801–5.672	0.1293

'I' + I, mass-forming + periductal infiltrative type.

TS also plays an important role in pyrimidine cascade, and is implicated to be associated with tumor characteristics in many cancers.[14,15,29,30] TS gene expressions in primary gastric cancer differ according to degree of tumor cell differentiation.[29] High-TS expression was associated with poor survival in patients with colon cancer, and increased TS activity was related to proliferation of tumor cells.[30] In primary liver cancer, Baba *et al.* reported TS activity was generally lower in HCC.[21] However, TS expression was not related to the prognosis of patients with HCC in our previous report.[16] In biliary tract cancer, TS concentration was higher in cancerous tissue than in noncancerous tissue, and the patients with high-TS concentration had a better disease-free survival rate.[23] However, there is a report suggesting that TS may not be a prognostic factor in gallbladder cancer.[24] In pyrimidine cascade, TS does not affect synthesis of deoxythymine monophosphate via thimidine, or metabolism of deoxythymine monophosphate.[8] Therefore, it seems that TS is insufficient to alter the DNA synthesis and tumor cell proliferation in IHCC.

In conclusion, the negative DPD expression was significantly associated with the increased tumor cell proliferation and poorer prognosis in IHCC patients. We expect that DPD expression may be a useful biomarker in the prediction IHCC patient prognosis.

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特集 外科臨床に必要な漢方治療の知識

術後障害に対する漢方治療

Beneficial effects of Kampo medicine for postoperative complications after digestive organ surgery

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漢方治療はエビデンスの蓄積と分子生物学的機序解明により適応疾患が拡大されつつある．当科では術後急性期においても，大建中湯，茵蔯蒿湯などの漢方薬剤を積極的に導入している．大建中湯は，腸管運動亢進・腸管血流増加作用により癒着性イレウス改善効果を示し，茵蔯蒿湯は減黄効果とともに肝切除術後の肝細胞保護効果などが期待される．今後，エビデンスのさらなる蓄積とともに術後障害に対しても広く臨床応用されることが期待される．

はじめに

漢方医学は，臨床的有用性が確認されるとともに西洋医学の分子生物学的手法などを用いた基礎的研究により作用機序解明も徐々に進み，今後さらに適応疾患の拡大が予想されている[1]．外科診療における漢方の位置づけとしては，これまではそのほとんどが手術により慢性化した病態に応用されてきたのが実状であった．しかしながら今後は，エビデンスの蓄積とともに術後を含めた急性期疾患においても積極的に適用することで十分に臨床効果が期待できると予想される．

そこで本稿では，消化器外科領域の術後障害に対してわれわれが実践している大建中湯，茵蔯蒿湯，六君子湯などを中心とした漢方治療の適応および有用性を示すメカニズムについて概説する．

I. 消化管手術の術後障害に対する漢方治療

1．大建中湯

大建中湯は乾姜，人参，山椒の3つの生薬に膠飴を加えた方剤であり，とくに山椒は本邦にしかない生薬である．消化管運動亢進，腸管血流増加，腸管過剰運動抑制，抗炎症作用など多彩な効果をもつなど作用機序の解明が進んでいる．実際の臨床現場では癒着性イレウスや麻痺性イレウス，過敏性腸症候群，クローン病などに対して広く使用されている日本独自の漢方方剤である．

1）消化管運動亢進作用

消化管運動亢進には筋層間神経叢におけるセロトニン受容体を介したアセチルコリン遊離作用，

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粘膜層におけるバニロイド受容体を介した直接作用，平滑筋層におけるモチリン分泌作用が関与し，平滑筋の中にある血管神経叢に作用して腸管平滑筋を刺激する[2]．成分別にみると，人参は腸管からの吸収の後に血流を介して消化管運動を亢進させ，山椒と乾姜は吸収後の血流を介さず腸管神経系に直接作用することから投与後30分以内と早期にその効果を発揮する．また，投与部位から肛門側の消化管に対して効果を発揮する．

2）腸管血流増加作用

大建中湯の腹部の冷えの改善効果は腸管運動の亢進のみならず，腸管の血流を介した効果であり，動物実験において消化管内に存在するCGRP(calcitonin gene related peptide：血管拡張因子)を介した作用や[3]，さらにこの血流増加作用がCGRPのみならずRAMP1(CGRP受容体)の発現増加が関与していることも解明されている[4]．

3）抗炎症作用

イレウスが長期に及んだ場合などにもっとも懸念される病態がBacterial translocationである．われわれはラット絶食モデルにおける大建中湯投与効果を検討したところ小腸粘膜組織の萎縮が有意に抑制された(図1)．さらに組織RNAのreal time RT-PCR解析にて炎症性サイトカインの有意な抑制が認められた(図2)[5]．これらの結果は，直接的な腸管の整合性の維持や炎症抑制効果によ

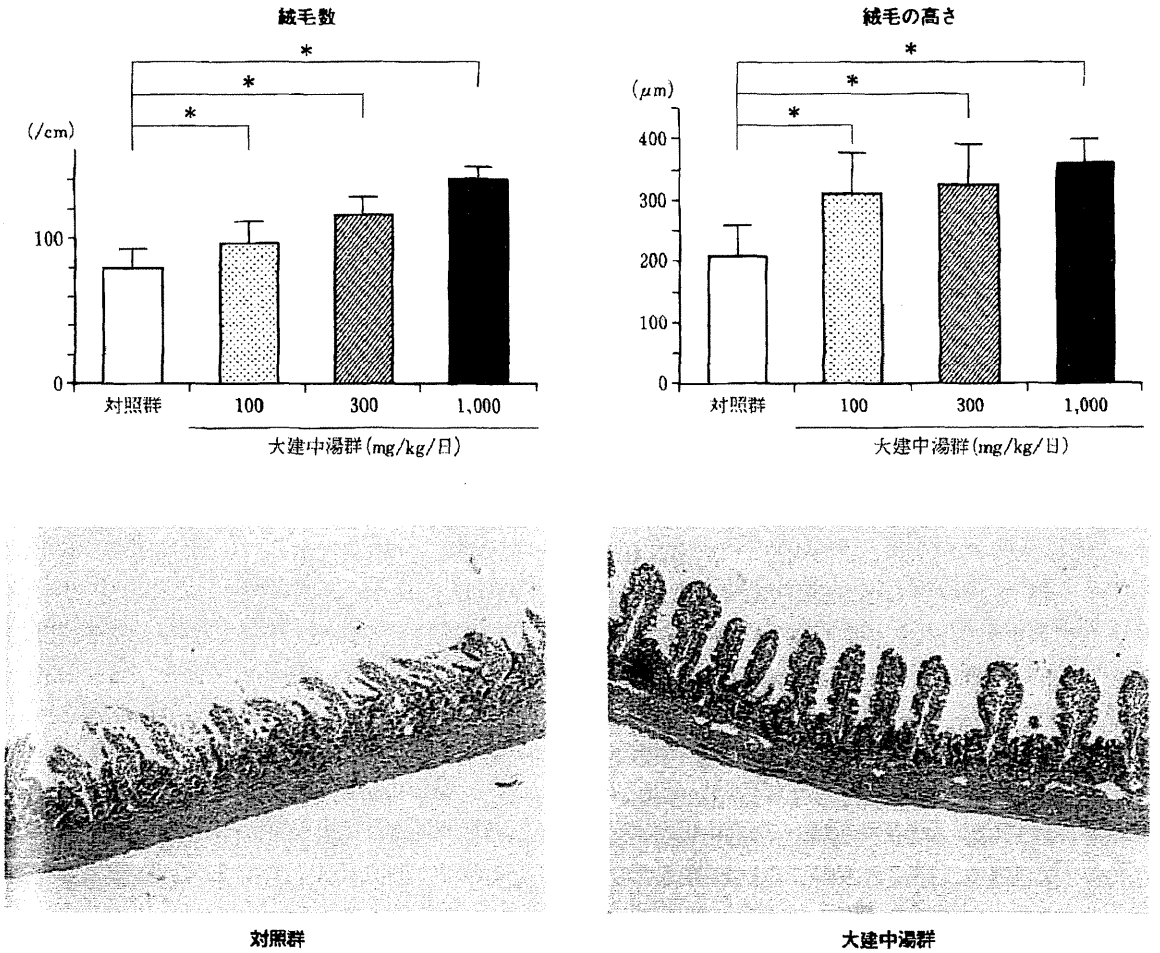

図1　大建中湯の小腸粘膜保護効果(文献5より改変)
＊P<0.05

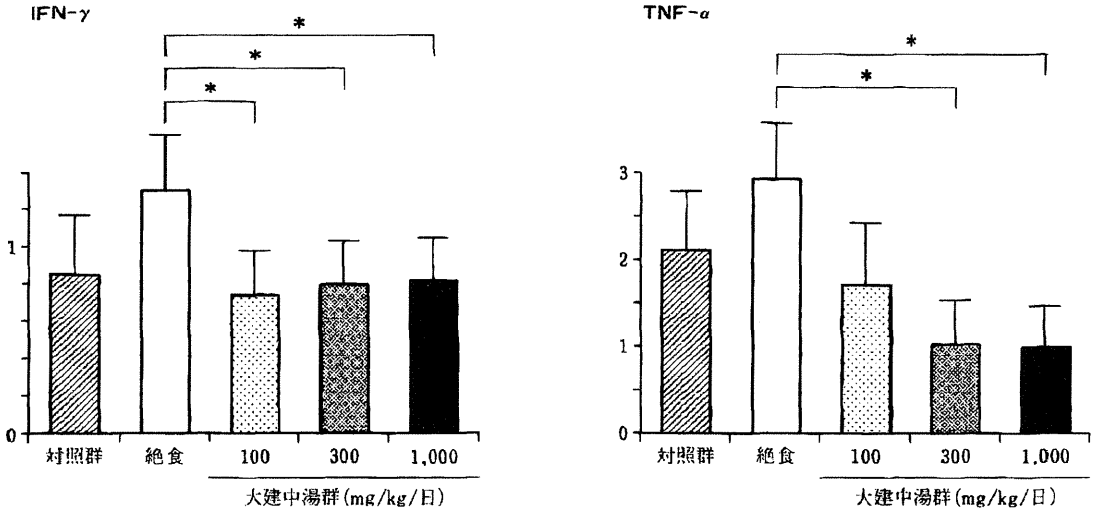

図2 大建中湯の炎症性サイトカイン抑制効果(文献5より改変)
＊P<0.05

りBacterial translocationを予防していることを示唆している.

4）イレウスに対する治療・予防効果

以上のような消化管運動亢進・血流改善作用や抗炎症作用を持つことから，イレウスに対する治療効果は早くから検討されその有用性が報告されてきた[6].

術後癒着性イレウス症例に対し1日7.5～15 gを経口および経鼻胃管，イレウス管から投与を行った群と非投与群の比較を行った報告では，在院日数，経口摂取までの期間が投与群で有意に短縮され，腹部単純X線写真での異常ガスの消失，イレウス管抜去，排便までの期間も改善され，手術までの期間は延長した．本剤投与によって手術までの時間的余裕が生まれ，さらに本剤無効例が手術適応となる可能性も考えられた[7]．大腸癌開腹手術症例に術後第1病日から7.5 gを経口投与した群と非投与群を比較検討した報告では，投与群において術後排ガスまでの期間，術後入院日数が有意に短縮され，在院期間内における腸閉塞の発症も少なかった．また術後1～2病日の早期に投与する方が入院期間短縮に有効であった[8]．さらに，大腸癌手術症例469例を同様に投与群，非投与群に分け，術後在院日数，医療費軽減効果を検討したところ，開腹手術でも腹腔鏡手術であっても術後入院日数は有意に短縮され，とくに腹腔鏡下手術で医療費節減効果がみられた[9]．イレウスとは異なるが，胃全摘術後に空腸嚢間置再建を行った患者に1日15 gを2週間投与した群と非投与群を比較検討すると，投与群で停滞に関する症状が減少し，^{111}Inや^{99m}Tcを用いた排出能試験でも排出の促進がみられ，空腸嚢運動の促進が確認されたとの報告もある[10].

小児外科領域においても，1日0.1～0.15 g/kgの投与量で術後イレウスや腹部手術後，Hirschsprung病，慢性便秘などのobstructive bowel diseaseの85%に有効であり，本剤が有効であれば投与続行し，無効であれば手術を考慮して精査を進めるとういう治療方針の推奨が報告されている[11].

安価な大建中湯を使用することで，イレウスの予防や腹部術後の回復を早める効果もあり，さらに在院期間を短縮することによって医療経済にも貢献すると考えられる.