にエトポシド＋CDDP＋5-FU (ECF 療法) をベースとして CDDP のかわりにオキサリプラチン, 5-FU のかわりにカペシタビンを用いた 4 群間での 2×2 の比較試験がおこなわれている.

わが国でおこなわれている第III相比較試験として, 日本臨床腫瘍研究グループ (JCOG) 消化器がん内科グループでは JCOG 9205[12]の結果, リファレンスアームとして残った 5-FU 持続静注 (5-FUci) 療法をコントロールとして, イリノテカン＋CDDP 併用療法および S-1 単独療法を比較している (JCOG 9912) が, イリノテカン＋CDDP の毒性と S-1 が経口薬である点を考慮し, 生存期間において 5-FUci と比較してイリノテカン＋CDDP の優越性と S-1 の非劣性を検証している. 2007 年に最終結果を報告予定であるが, JCOG 9912 の結果, イリノテカン＋CDDP の優越性と S-1 の非劣性がともに示された場合には, 両療法ともダブルスタンダードと結論されるであろう. 一方, わが国では S-1 単独療法がコミュニティースタンダードとして広く用いられており, S-1 単独療法をコントロールとして 5-FU/LV, S-1＋CDDP, S-1＋イリノテカンとの比較試験もおこなわれている. JCOG 9912 の結果, S-1 が標準的治療の一つとなった場合には, 上記の S-1 を規準とした併用療法のいずれかの試験においてポジティブな結果が期待される. 逆に, JCOG 9912 の結果, S-1 がスタンダードとならなかった場合には, 上記の S-1 をベースにした他剤との併用療法の比較試験の意義は小さくなることが懸念される.

上記の現在進行中の第III相比較試験では非劣性を検証するものが多く, 簡便性や安全性が高まることは期待されるが, 延命効果がしめされなければ真の意味での進歩とはいいがたい. 大腸癌では分子標的治療薬が導入されたことにより, 大きな進歩と新たな展開が開かれた. 胃癌に対する化学療法にも分子標的治療薬の導入が待たれるが, 上記の比較試験の結果で世界的にコンセンサスの得られた標準的治療が確立され, そのうえに分子標的治療薬の追加が検証されることが望まれる.

しかし, すでに実地医療では新規薬剤の第 I / II 相試験の結果が一次治療だけでなく二次治療以降にも導入されるようになり, 最近では切除不能・再発胃癌に対する全身化学療法の生存期間の中央値は 10～12ヵ月になりつつあるなど, 1990 年代とくらべれば切除不能・再発胃癌に対する全身化学療法の治療成績は向上したといえる. 従来, 胃癌は化学療法が効かないとされてきたが, 胃癌に対して有効な薬剤が多く開発され, 患者の全治療期間中で有効な薬剤を使いこなすことによって生存期間を得られるかについて, 胃癌化学療法全体の治療戦略の構築が必要であると思われる.

3 ▌大腸癌における化学療法

大腸癌においては, イリノテカン, オキサリプラチン, bevacizumab, cetuximab などの分子標的治療薬を含めた新規薬剤の開発により, 切除不能・再発症例では比較試験の結果で明らかに延命効果が示され, 術後補助化学療法においては無再発生存期間の延長が得られるようになった. 詳細については他稿を参照していただきたい.

4 ▌膵癌における化学療法

膵癌に対する化学療法は, 5-FU 単独が標準的治療と考えられてきたが, 治療効果が小さいため無治療となる症例も少なくなかった. ゲムシタビン (GEM) は 5-FU との疼痛, 体重, パフォーマンスステータスを指標とした clinical benefit response (CBR) をプライマリーエンドポイントとした比較試験において, CBR だけでなく, 生存期間においても有意に良好な成績を示した[13]. これを受けて, わが国でも遠隔転移を有する膵癌に対する標準的治療として広く GEM が用いられるようになった. 近年, GEM と 5-FU, CDDP, イリノテカンなどの抗癌剤の併用療法が開発されたが, いずれの併用療法も GEM 単独との比較試験において有意な延命効果を示すことができなかっ

た．一方，GEMは通常1,000 mg/m²を30分で点滴静注されるが，細胞内濃度を考慮して1,500 mg/m²を150分かけて静注する（10 mg/m²/分，fixed dose rate-GEM：FDR-GEM）方法とGEM 2,200 mg/m²を30分で点滴静注療法との無作為化第II相試験では，FDR-GEMのほうが良好な成績を示した[14]．これを受けて，FDR-GEMにオキサリプラチンを併用すると，GEM単独に比較して生存期間が長い傾向が示された．すでに，欧米ではこの併用療法がコミュニティースタンダードとして用いられつつあり，追試がおこなわれている．また，上皮成長因子レセプター（epidermal growth factor receptor：EGFR）の阻害薬であるerlotinibとGEM単独の比較試験の結果，生存期間の差はわずか（5.9ヵ月，6.4ヵ月）であるが，有意な延命効果が示された（ハザード比＝0.81，p=0.025）[15]．このことは，はじめてGEMよりすぐれた治療法が証明されたことと，分子標的治療薬が膵癌にも有効性を示した点において意義が大きいと思われる．さらに，bevacizumab，cetuximabを併用する比較試験も進行中である．また，GEMを膵癌の術後補助化学療法として用いることにより，まだ中間解析ではあるが，無病生存期間の延長が得られたと報告されている[16]．

おわりに

本稿では，食道癌，胃癌，大腸癌，膵癌について記載したが，食道癌・胃癌では分子標的治療薬を含めた新薬の大規模な比較試験の結果による信頼性の高いエビデンスがなく，大腸癌などと比較するとこれらの開発が遅れているといわざるを得ない．とくに，胃癌の新薬開発においては世界的にもわが国の果たすべき役割は大きいが，一方で，すべての癌種において世界的には大規模試験がおこなわれつつある．わが国（国内の各グループ，施設）のイニシアティブを主張するよりも，世界的な視野に立って，最も早く，効率的に治療成績を向上させるために，われわれがおこなわなければならないことをしっかりと見つめて，更なる進歩に貢献できるよう努力すべきであると思われる．

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Original article

Weekly paclitaxel as second-line chemotherapy for advanced or recurrent gastric cancer

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Abstract

Background. **Paclitaxel scheduled every 3 weeks has shown a response rate of ~20% for gastric cancer, with modest hematological toxicity. Weekly administration of paclitaxel in patients with breast or ovarian cancer has shown equivalent efficacy and milder toxicity compared with an every-3 week schedule. We investigated, retrospectively, the antitumor effects and toxicity profiles of weekly paclitaxel for patients with metastatic or recurrent gastric cancer in clinical practice.**

Methods. **In 38 patients who had metastatic or recurrent histologically confirmed gastric cancer and a history of one prior chemotherapy regimen, other than paclitaxel or docetaxel, paclitaxel (80 mg/m^2) was administered weekly, three times every 4 weeks, with short-term premedication.**

Results. **All 38 patients had had prior chemotherapy that included 5-fluorouracil, the fluoropyrimidine anticancer drug S-1, or cisplatin. The median number of courses in the present regimen was 6 (range, 1–44+). Dose intensity was 55 mg/m^2 per week, corresponding to 92% of the planned dose (60 mg/m^2 per week). The overall response rate was 24% (6/25) in measurable lesions, and pleural effusion and ascites disappeared in 2 of 7 patients (29%) and in 3 of 21 patients (14%), respectively. Median survival time was 151 days from the commencement of this treatment, with a median follow-up period of 260 days. Grade 3 or 4 leukopenia and neutropenia were observed in 11 (29%) and 12 (32%) patients, respectively. Seven patients (18%) died within 30 days of the last administration of paclitaxel.**

Conclusion. **Weekly paclitaxel seems to be active as second-line chemotherapy against metastatic and recurrent gastric cancer. Further study is needed to confirm the efficacy and safety of weekly paclitaxel.**

Key words Gastric cancer · Weekly paclitaxel · Second-line chemotherapy

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Introduction

Gastric cancer remains as one of the major causes of death from cancer worldwide. Despite a markedly improved survival trend through early detection and curative surgery, approximately 50000 deaths from gastric cancer occurred in Japan in 1997 [1]. Although patients with metastatic and recurrent gastric cancer still have a poor prognosis, no standard chemotherapy regimen has been established, even as the first-line.

Paclitaxel is an antitumor agent active against various kinds of malignancies. Because paclitaxel is known to be a cell-cycle-specific agent [2,3], basic research has suggested that prolongation of exposure might enhance its cytotoxic effects. In a phase I study of weekly paclitaxel, a regimen of 80 mg/m^2 in a 3-weeks-on and 1-week-off schedule was recommended [4]. In this phase I study, a weekly paclitaxel regimen (1-h infusion) produced objective tumor regression in patients previously treated with paclitaxel on a once-every-3-weeks schedule. Recently, successful results have been achieved using a regimen of weekly paclitaxel in patients with breast cancer and ovarian cancer. Hematological toxicity caused by a weekly 3-h infusion schedule has been reported to be milder, with equivalent activity, compared with a schedule of one 24-h infusion every 3 weeks [5].

Paclitaxel is recognized as one of the active cytotoxic agents for gastric cancer [6–9]. A weekly paclitaxel regimen has become popular in Japan, mainly as second-line chemotherapy, because of its milder hematological toxicity compared with a once every 3 weeks schedule of paclitaxel. However, only a few trials of weekly paclitaxel in patients with gastric cancer have been reported [10,11]. In the present retrospective study, we investigated the potential and safety of this more dose-dense weekly paclitaxel regimen in patients with pretreated gastric cancer.

Patients and methods

Patients

The subjects of this study consisted of 38 patients with metastatic or recurrent gastric cancer treated weekly with paclitaxel between September 2002 and September 2004 at the Shizuoka Cancer Center, Shizuoka, Japan. The recruitment criteria were as follows: (1) histologically proven adenocarcinoma of the stomach; (2) history of one prior chemotherapy regimen not involving paclitaxel or docetaxel; (3) age, 75 years or less; (4) performance status of 2 or less on the Eastern Cooperative Oncology Group scale; (5) adequate bone marrow, hepatic, and renal functions; (6) no other serious disease; (7) and oral or written informed consent given before the commencement of treatment.

Treatment methods

The treatment schedule comprised an intravenous infusion of paclitaxel at 80 mg/m^2 in 250 ml normal saline over 1 h, repeated weekly three times for 4 weeks. Short-term premedication for paclitaxel-associated hypersensitivity reactions was used: dexamethasone, 8 mg; diphenhydramine, 50 mg; ranitidine, 50 mg; and granisetron, 3 mg were administered 30 min before the paclitaxel treatment. This treatment was repeated until disease progression or prohibitive toxicity, usually on an outpatient basis. In the event of serious hematological toxicity, treatment was suspended until recovery. If grade 4 hematological or grade 3 or 4 nonhematological toxicity occurred, the dose of paclitaxel was reduced to 60 mg/m^2.

Response and toxicity assessments

Tumor measurements for response assessment in patients with primary lesions were made every 1 to 2 months by computed tomography (CT) and endoscopy. Objective responses in measurable metastatic lesions were evaluated according to the response evaluation criteria for solid tumors [12]. Survival time was calculated from the date of the commencement of paclitaxel treatment to the date of death or the last confirmation of survival. Symptomatic toxicity and laboratory data were monitored every week at the outpatient clinic. Toxicity was evaluated according to the National Cancer Institute common toxicity criteria (version 2).

Results

Patient population

Of 51 patients with advanced or recurrent gastric cancer treated with weekly paclitaxel as second-line chemotherapy, 38 patients fulfilled the recruitment criteria, and were included in this study. The 13 excluded patients had severe medical complications: 4 patients were aged 76 years or more, and 9 were performance status 3 or more.

Patient characteristics are presented in Table 1. Most (63%) were male, and the median age was 63 years. Twenty seven (71%) had a performance status of 0 or 1. All patients had had prior fluorouracil-based chemotherapy as first-line chemotherapy. Seven patients had pleural effusion and 21 had ascites. The number of metastatic organs (including liver, lymph node [LN], peritoneum, lung and bone), was one organ in 15 patients, two organs in 14 patients, and three organs or more in 9 patients.

Dose intensity

The total number of administrations of paclitaxel was 364. The median number of courses per patient was 6 (range, 1–44+). Dose intensity was calculated as 55 mg/m^2 per week, which corresponded to 92% of the planned dose. This treatment was stopped in 35 patients, because of disease progression in 31 patients,

Table 1. Patient characteristics

No. of patients	38
Sex	
Male	24
Female	14
Age (years)	
Median	63
Range	51–73
Performance status	
0	12
1	15
2	11
Prior chemotherapy	
S-1	29
MTX + 5-FU	4
S-1 + CDDP	2
5-FU	1
5-FU + CDDP	1
UFT	1
Histology	
Intestinal	13
Diffuse	21
Unknown	4
Sites of metastasis[a]	
Liver	10
Lymph node	21
Peritoneum	29
Lung	2
Bone	4
Pleural effusion	7
Ascites	21

[a]Some patients had metastases at multiple sites

Table 2. Toxicity

	Grade 1	Grade 2	Grade 3	Grade 4	Percentage ≥ grade 3
Leukocytes	7	5	9	2	29
Neutrophils	4	6	6	6	32
Platelets	2	2	3	0	8
Nausea	6	2	1	—	3
Vomiting	4	0	0	0	0
Anorexia	7	8	1	0	3
Diarrhea	4	1	1	0	3
Neuropathy—motor	0	0	1	0	3
Neuropathy—sensory	0	9	1	0	3
Edema	3	1	1	0	3
Allergic reactions	3	0	0	0	0

infection in 1, neuropathy in 1, refusal of further treatment in 1, and non-cancer death related in 1 patient.

Toxicity

Toxicity data are presented in Table 2. Of hematological toxicities, 11 patients (29%) experienced leukopenia of grade 3 or 4 and 12 (32%) had neutropenia of grade 3 or 4. Of nonhematological toxicities, 1 patient (3%) had grade 3 nausea and anorexia. Severe neuropathy was seen in 1 patient (3%) and no allergic reaction was seen in any patient. Seven patients (18%) died within 30 days of the last administration of paclitaxel. The reasons for these early deaths were disease progression in 3 patients; death of other causes (acute myocardial infarction in 1 and heart failure in 1); perforation of the esophagus in 1 (due to an inserted expandable metallic stent for esophageal stenosis caused by mediastinal lymph-node metastasis); and sepsis with grade 4 neutropenia in 1.

Responses and survival

Twenty-five of the 38 patients were assessable for response (13 patients did not have measurable disease). Of these 25 patients, 6 (24%) experienced a partial response (Table 3). The details of the responders are shown in Table 4. Pleural effusion disappeared in 2 of 7 patients (29%) and decreased in 1 of 7 patients (14%) after treatment with paclitaxel. Ascites disappeared in 3 of 21 patients (14%) and decreased in 2 of 21 patients (10%). The median follow-up period was 260 days. Survival data were updated in February 2005. The median survival time was 151 days after the initiation of weekly paclitaxel therapy (Fig. 1). The median time to progression was 64 days (Fig. 2). Twenty-one patients (55%) had no further chemotherapy after disease progression following paclitaxel therapy. Nine patients had

Table 3. Response

n	PR	SD	PD	NE	RR
25	6	4	14	1	24%

PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; RR, response rate

irinotecan-containing regimens, 2 had a 5-fluorouracil (5-FU)-based regimen, 1 had intraperitoneal administration of cisplatin (CDDP), and 1 had palliative radiation therapy at the primary site.

Discussion

Recently, weekly paclitaxel has been commonly used in second-line or higher chemotherapy. In the present retrospective study, all 38 patients had a history of prior chemotherapy at our institution. Moreover, 9 patients (24%) had three involved metastatic sites, and 4 (11%) had bone metastases. Seven patients had pleural effusion and 21 had ascites. To clarify the activity and toxicity of paclitaxel as second-line chemotherapy in a clinical practice setting, the recruitment criteria for this study allowed patients to have pleural effusion and/or ascites.

We found weekly paclitaxel therapy to be well tolerated by most patients, considering the dose intensity used and its toxicity profile. Previous reports showed that grade 3 or 4 neutropenia occurred in 37%, and grade 4 neutropenia in 67% of patients treated with paclitaxel on an every-3-weeks regimen [7,9]. In our study, 29% and 32% of patients had severe leukopenia and severe neutropenia, respectively. On the basis of these results, a weekly regimen of paclitaxel seems to be less toxic than an every-3-weeks regimen in terms of

Table 4. Details of responders treated with weekly paclitaxel

Patient no.	Age (years)	Sex	Metastatic site	Reduction ratio	Total number of administrations	Response duration (days)
1	68	M	Abdominal wall	61%	44	413
2	57	F	LN, peritoneum	32%	6	41
3	65	F	LN, liver	37%	4	50
4	72	M	Liver, LN, peritoneum	36%	5	99
5	71	M	Liver, LN, lung	35%	13	119
6	66	M	LN, abdominal wall, peritoneum	70%	11	328

LN, lymph node

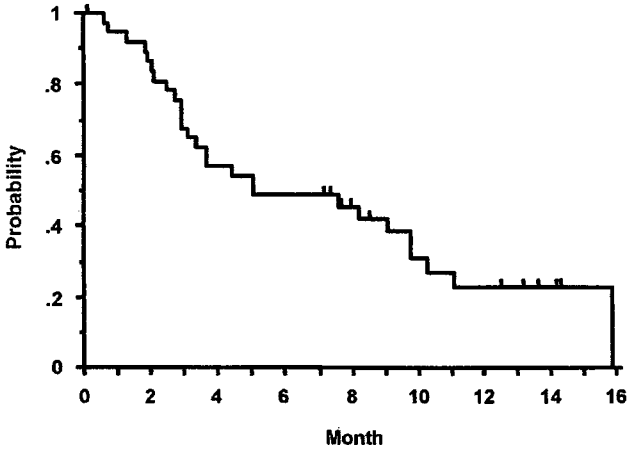

Fig. 1. Kaplan–Meier analysis of overall survival. Median overall survival time was 151 days

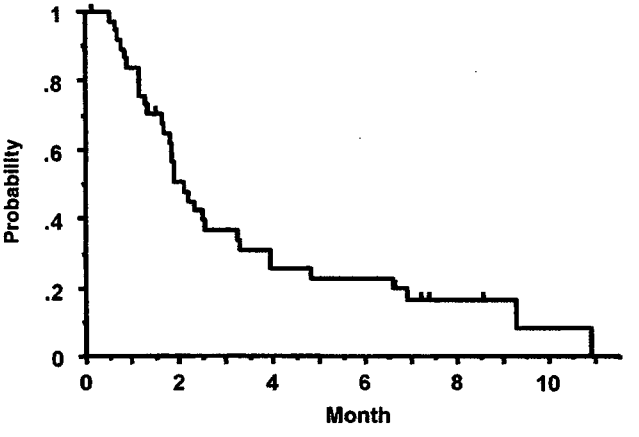

Fig. 2. Kaplan–Meier analysis of time to progression. Median time to progression was 64 days

hematological toxicity. Furthermore, nonhematological toxicities were generally mild.

Seven patients died within 30 days of the last administration of paclitaxel. The causes of the deaths in our study were disease progression in three patients, other medical diseases in two patients, complications due to a metallic stent in one, and treatment-related sepsis in one. These patients had had a severe medical condition or poor oral intake and poor performance status at the last administration of paclitaxel. Therefore, we have to take care regarding the patient's condition and consider cautiously the indications for the administration of paclitaxel.

With weekly paclitaxel therapy, we observed a response rate of 24% in 25 patients with measurable metastatic lesions. Disease stabilization was observed in 40% (10/25). Ascites and pleural effusion decreased or disappeared in 24% (5/21) and 43% (3/7), respectively. Direct comparison of response rates from one trial to another is inherently difficult, given that studies often differ with respect to entry criteria and population characteristics. Nevertheless, overall response rates of 8%–27% have been reported in other trials of single-agent paclitaxel administered for gastric cancer at doses of 210 mg/m^2 by 3-h infusion every 3 weeks for gastric cancer [6–9]. Therefore, our response results are within the range observed in other trials, of paclitaxel given every 3 weeks.

On the basis of previously reported data, the median survival time for metastatic or recurrent gastric cancer is about 7 to 9 months [13–17] with first-line chemotherapy. In the present retrospective study, the median overall survival time after the administration of paclitaxel was about 5 months. These data are the same as the previously reported data [10] for weekly paclitaxel. Our results suggest that weekly paclitaxel may have similar activity to paclitaxel given on a 3-week schedule for patients with metastatic or recurrent gastric cancer after prior therapy.

In conclusion, weekly paclitaxel as second-line chemotherapy was tolerated and demonstrated activity against metastatic and recurrent gastric cancer. How-

ever, its administration in practice must be decided with caution in patients in poor condition. The Japan Clinical Oncology Group (JCOG) is now conducting a randomized phase II trial of weekly paclitaxel versus best available 5-FU for second-line chemotherapy for gastric cancer with peritoneal dissemination.

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●原 著●

切除不能・再発胃癌に対するWeekly Paclitaxel（PTX）の有効性と副作用の検討

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Weekly Administration Regimen of Paclitaxel (PTX) in Patient with Inoperable or Recurrent Gastric Cancer: Takayuki Kii, Hiroya Takiuchi, Masahiro Gotoh, Shinichiro Kawabe, Shunsuke Ohta, Toshimitsu Tanaka, Shin Kuwakado, Hitoshi Nishitani and Ken-ichi Katsu (*Second Dept. of Internal Medicine, Osaka Medical College*)

Summary

Paclitaxel is one of the new drugs against advanced/recurrent gastric cancer. We report its efficacy and toxicity with weekly administration for advanced/recurrent gastric cancer. We administered 26 patients (postoperative/non-operation=9/17) PTX 80 mg/m^2 by 1-hour intravenous infusion once a week for 3 weeks followed by one-week rest. Median PTX administrations were 2.0 cycles (range: 1-22). Characteristics of the patients were median age of 62 (range: 37-78) and PS 0/1/2: 2/17/7, male/female: 18/8. Over grade 3 toxicities did not occur. The overall response rate was 14.3%, and the non-PD rate was 66.8%. Median time to treatment failure was 61 days and median survival time was 221 days. These results suggest that weekly PTX has modest activity with a favorable toxicity profile in patients with advanced/recurrent gastric cancer, and so this regimen may thus might be recommended in an outpatient treatment setting. **Key words**: Weekly paclitaxel, Advanced/recurrent gastric cancer

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要旨 前治療の有無を問わない切除不能・再発胃癌に対するweekly PTXの有効性と副作用についてretrospectiveな検討を行った。対象患者は26例，切除不能胃癌が17例，手術後の再発胃癌が9例，男性18例，女性8例であり，平均年齢65歳(37～78歳)，初回治療2例，二次治療17例，三次治療以降は7例であった。投与サイクルの中央値は2.0サイクルで，測定可能病変のある症例は21例であった。そのうちCR 0%，PR 14.3% (3/21)，NC 52.4% (11/21)，PDは33.3% (7/21)で，non-PD率は66.7% (14/21)であった。治療成功期間（PTXの治療開始日から最終投与日）の中央値（TTF）は61日，生存期間中央値（MST）は221日であった。また，grade 3以上の副作用は認められず，副作用も軽度であることから，長期にわたって外来投与可能であることが示唆されたと考える。

はじめに

切除不能・再発進行胃癌に対する化学療法においてpaclitaxel（PTX）は，phase I studyにおける推奨投与量は210 mg/m^{2}で3週間ごとと決定された[1]。しかし，この治療では白血球減少，好中球減少，感覚性神経障害などの副作用の出現頻度が比較的高く，様々な投与法が検討されている。なかでも1週間ごとの少量投与(weekly PTX)は，海外での臨床試験において乳癌，卵巣癌などで3週間ごとの投与に比較して毒性も軽く，また同等の抗腫瘍活性を有する可能性が示唆されている[2,3]。

今回，前治療の有無を問わない切除不能・再発胃癌に対するweekly PTXの有効性と副作用についてretrospectiveな検討をしたので報告する。

I．対象と方法

1．対象症例

2001年7月～2003年10月に下記の基準を満たす患者26例を対象とした。①前治療の有無は問わない，②組織学的に胃癌と確認された症例，③80歳以下である，④主

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要臓器（骨髄，心，肝，腎）の機能が保持されている，⑤PSは3以下。

患者背景は男性18例，女性8例であり，年齢中央値62歳（37～78歳）であった。PSは0が2例，1が17例，2が4例，3が3例で切除不能胃癌17例，手術後の再発胃癌が9例であった。評価可能病変（重複あり）として原発巣17例，肝転移10例，リンパ節転移6例，副腎転移2例であり，腹水を有する症例14例，骨転移を有する症例2例が含まれていた。そのなかで標的病変を有する症例は21例で，腹水のみの症例が5例であった。肉眼型は2型4例，3型9例，4型12例，不明1例で，病理組織型はpap 1例，tub1 1例，tub2 6例，por/sig 16例，ade＋scc 1例，不明が1例であった（表1）。

前治療としては，first-line chemotherapyとしてweekly PTXを施行した症例が2例，second-line chemotherapyとして施行した症例が17例であった。second-line chemotherapyとして施行した17例のfirst-line chemotherapyの内訳はTS-1が10例，TS-1＋CPT-11が5例，5-FUが1例，CDDP＋CPT-11が1例であった。また，third-line chemotherapyとして施行した症例は6例で，first-line chemotherapyとしてTS-1が2例，TS-1＋CPT-11が1例，UFTが1例，CDDP＋CPT-11が1例，5-FU＋LVが1例，second-line chemotherapyとしてTS-1が2例，TS-1＋CPT-11が1例，Iressa（治験として）が1例，CDDP＋CPT-11が1例で，fourth-line chemotherapy以上も1例含まれていた（表2）。

対象症例26名に対してweekly PTX 80 mg/m²を点滴静注にて1時間かけて投与し，3週連続投与（day 1, 8, 15），1週休薬で施行し，これを1サイクルとした。PTX投与前のpremedicationとしてdexamethasone 20 mg (div)，diphen hydramine 50 mg (po)，ranitidine 50 mg（div）を施行した（図1）。

続けて，1クール終了ごとに血液検査で腫瘍マーカー（CEA，CA 19-9）を計測し，適宜CT検査，上部消化管内視鏡検査，胃透視などを施行した。治療効果に関して，抗腫瘍効果は胃癌取扱い規約（第13版），副作用はNCI-CTC（日本語訳JCOG版第2版）によって判定した。治療成功期間（PTXの治療開始日から最終投与日：time to treatment failure: TTF）の中央値，全生存期間の中央値（median survival time: MST）はKaplan-Meier法を用いて算出した。また，奏効期間はPR確認後から最終投与日までとした。

表1　患者背景（n＝26）

男性/女性	18/8
年齢（中央値）	62
(Range)	(37～78)
PS　0/1/2/3	2/17/4/3
手術（＋/－）	9/17
肉眼型	
1/2/3/4/unknown	0/4/9/12/1
組織	
分化型/未分化型/ade＋scc/不明	8/16/1/1
標的病変	
原発/肝臓/リンパ節/副腎	17/10/6/2
非標的病変	
腹水/骨	14/2

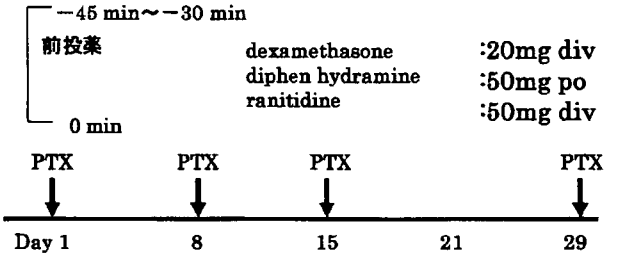

図1　治療方法
PTX 80 mg/m²を1時間で点滴静注 day 1, 8, and 15, に行い，day 21は休薬とし，これを1コースとした

表2　前治療

PTX		First-line		Second-line	
First-line	2	TS-1	10		
Second-line	17	TS-1＋CPT-11	5		
		5-FU	1		
		CDDP＋CPT-11	1		
Third-line以上	7	TS-1	2	TS-1	2
		TS-1＋CPT-11	1	TS-1＋CPT-11	1
		UFT	1	Iressa	1
		CDDP＋CPT-11	1	CDDP＋CPT-11	1
		5-FU＋LV	1	MF	1
		Many	1		

median numbers of PTX administrations　2.0 cycles (range: 1～22)

表 3 Efficiency（胃癌取扱い規約第13版による）

		Rate (%)
CR	0/21	0
PR	3/21	14.3
NC	11/21	52.5
PD	7/21	23.3

表 5 Toxicity (NCI-CTC)

grade	0	1	2	3	4	Rate (%)
白血球減少	17	1	8	0	0	38
貧血	2	1	1	0	0	7
脱毛	19	6	0	0	0	27
吐気	7	5	2	0	0	27
末梢神経障害	5	5	0	0	0	19
下痢	1	0	1	0	0	4

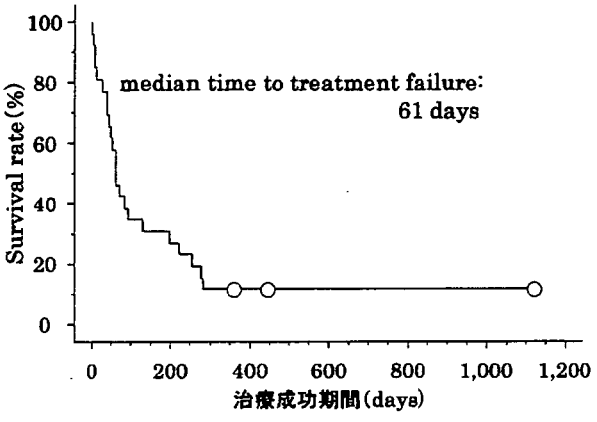

図 2 治療成功期間

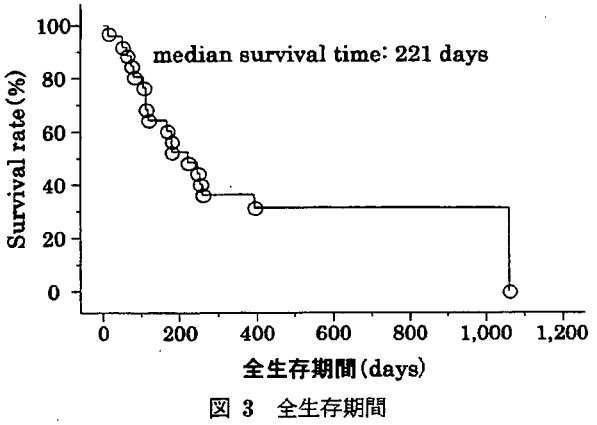

図 3 全生存期間

II. 結 果

PTXの投与サイクルの中央値は2.0サイクルで，測定可能な標的病変のある症例は21例であった。そのうちCR 0%，PR 14.3% (3/21)，NC 52.4% (11/21)，PD 33.3% (7/21) であった。奏効率は14.3% (3/21)，non-PD率66.7% (14/21) であった（表3）。PR例の詳細は男性2例，女性1例であり，奏効期間最長は1,105日，治療成功期間最長は968日であった（表4）。また，半年以上（6サイクル以上）の長期投与は27% (7/26) 症例に可能であった。治療成功期間の中央値（TTF）は61日（図2）で，生存期間中央値（MST）は221日であった（図3）。

表 4 PR症例（3例）

年齢	性別	奏効部位	奏効期間（日）	治療成功期間（日）
75歳	女性	胃，リンパ節	168	278
52歳	男性	胃	30	109
78歳	男性	胃，肝臓	968	1,105

grade 1～2の有害事象は白血球数減少38% (10/26)，脱毛27% (7/26)，悪心・嘔吐27% (7/26)，末梢神経障害19% (5/26)，下痢7% (2/26)，貧血4% (1/26)，全身倦怠感4% (1/26) であり，grade 3以上の副作用は認められなかった（表5）。

III. 考 察

1990年代に入り進行・再発胃癌に対する新規抗癌剤としてTS-1，CPT-11，taxotere，PTXが登場しfirst-line，second-lineを問わず様々な検討が行われている。その一つであるtaxane系のPTXは，西洋イチイ (Pacific yew: 学名 *Taxus baccata*) の針葉の抽出物を前駆体とした半合成品で，微小管重合を促進することによって細胞分裂周期をG_2/M期で阻害する。国内での第I相試験で，推奨投与量は210 mg/m^{2}で3週間ごとと決定された[1]。胃癌の第II相試験における前化学療法歴を有する患者の奏効率は27%で，これまで報告されている二次治療としてのTS-1単独療法，MTX+5-FU時間差療法を上回る[4-6]。しかし，3週間ごと投与は白血球減少，好中球減少，貧血，脱毛，嘔気，下痢，感覚性神経障害などの副作用の出現が問題となっており，投与法の工夫が試みられている。なかでもweekly PTX投与法は海外で乳癌，卵巣癌などにおいて3週間ごとの投与に比較して毒性も軽く，また同等の抗腫瘍活性を有する可能性が示唆されている[2,3]。現在，胃癌に対する標準的化学療法は存在せず，第一次治療として5-FU系薬剤を中心とした治療が選択されることが多い[7,8]。また，作用機序で5-FU系薬剤やCDDPとは異なっており，これらの薬剤に対して抵抗性となった患者にも効果が認められ，交叉耐性がないとされている[9]。よって現状では，PTXはsecond-line以降のkey drugの一つと目されている。

今回のretrospectiveな検討では奏効率は14.3% (3/21) と低いが，non-PD率は66.7% (14/21) と高く，

weekly PTX投与日からの全26例のTTFは61日，MSTは221日であった。そのなかで6サイクル以上の長期投与が行われた症例は27%（7/26）で，なかには最長22サイクル投与中の患者もいた。副作用に関してはgrade 3以上の副作用は認められなかった。この結果からweekly PTXは非常に忍容性の高い治療と考えられる。新井らもweekly PTXのsecond-line以降のprospectiveな検討を行い，奏効率23.5%，TTFは77日であり，grade 4の副作用は血液毒性として好中球減少1例(2%)，神経障害，ふらつきによる治療中止3例(7%)を認めたが治療関係死はなく，重篤な有害事象も極めて低頻度であったと報告している[10]。

以上より，weekly PTXはsecond-line以降においても比較的安全に外来で投与可能なレジメンと考えられ，今後，切除不能・再発進行胃癌に対する治療法を組み立てる上で重要な選択肢の一つになると思われる。

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In-vitro differential metabolism and activity of 5-fluorouracil between short-term, high-dose and long-term, low-dose treatments in human squamous carcinoma cells

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Although continuous infusion of 5-fluorouracil (5-FU) has been clinically demonstrated to be superior to bolus administration, the mechanistic difference between the treatments still remains unclear. Here, we investigated *in vitro* whether there were any differences in the metabolism and activity of 5-FU between these schedules. To simulate bolus and infusional treatments of 5-FU, HST-1 human squamous carcinoma cells were treated with short-term, high-doses and long-term, low-doses so that the area under the curve (AUC) of 5-FU became equivalent between both schedules, and compared the cytotoxicity, fluorinated RNA (F-RNA) levels, 5-fluorodeoxyuridine monophosphate (FdUMP) content and thymidylate synthase (TS) activity. F-RNA and FdUMP were measured by capillary gas chromatography–mass spectrometry and competitive ligand-binding assay, respectively. The [^{3}H]FdUMP binding site in TS was determined as an index of the amount of TS using the radio-binding assay. Long-term, low-dose treatment of 5-FU was found to be 1.3–1.7 times more cytotoxic than the short-term, high-dose treatment. F-RNA content increased as the AUC of 5-FU was increased and was 2–4 times significantly higher in the cells treated with the long-term, low-dose than those with the short-term, high-dose schedule, indicating that the levels of F-RNA are AUC and schedule dependent. In contrast, there were no significant differences in FdUMP levels, TS activity and TS inhibition rate between the schedules. These data suggest that the superior activity of 5-FU administered long-term, low-dose over short-term, high-dose could be explained by more 5-FU incorporated into RNA during a long-term, low-dose exposure, thus providing a strategic rationale for the clinical advantage of continuous infusion over bolus administration. *Anti-Cancer Drugs* 17:439–443 © 2006 Lippincott Williams & Wilkins.

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Keywords: 5-fluorodeoxyuridine monophosphate, 5-fluorouracil, bolus, continuous, fluorinated RNA, thymidylate synthase activity

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Introduction

5-Fluorouracil (5-FU) is effective either singly or in combination with other anti-cancer drugs against a variety of human solid tumors. This drug has multiple mechanisms of action including (a) inhibition of thymidylate synthase (TS) by 5-fluorodeoxyuridine monophosphate (FdUMP), (b) incorporation of 5-fluorouridine triphosphate (FUTP) into RNA, and (c) incorporation of 5-fluorodeoxyuridine triphosphate (FdUTP) into DNA, thereby disrupting DNA synthesis as well as RNA/DNA function. Because of these differential mechanisms, 5-FU exhibits clinically different maximal tolerated doses and different dose-limiting toxicity according to the administration schedules (e.g. bolus or infusion) [1]. Continuous infusion of 5-FU has been shown to be clinically superior to bolus administration in anti-tumor activity [2–4]. However, the mechanistic differences between these treatments still remain to be clarified. It has been shown *in vitro* that short-term treatment with 5-FU produced resistance via decreased incorporation of FUTP into RNA, while repeated prolonged exposure to 5-FU produced resistance via rapid recovery from TS activity [5], suggesting that 5-FU administered in bolus and continuous fashions may preferentially cause RNA- and DNA-directed (inhibition of TS) cytotoxicity, respectively. Clinically, TS inhibition in cancer tissues has been shown to be significantly higher in patients treated with infusional 5-FU than those treated with the bolus administration [6]. However, these experiments were not conclusive because the RNA- and DNA-directed parameters indicative of 5-FU activity had not been directly measured and compared between these schedules.

In this study, we investigated the mechanisms whereby bolus or infusional schedules of 5-FU administration cause cytotoxic effects by directly measuring the parameter including 5-FU incorporated into RNA (F-RNA), FdUMP and TS inhibition using HST-1 human squamous carcinoma cells.

Materials and methods

Cell culture and chemicals

A clonally isolated subline of HST-1 human tongue squamous carcinoma cells [7] was maintained in DMEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% heat-inactivated FBS (Gibco/Life Technologies, Grand Island, New York, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco/Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2/95% air. 5-FU was kindly provided by Kyowa Hakko (Tokyo, Japan). Immediately before use, 5-FU was dissolved in a culture medium. [^{3}H]6-FdUMP was purchased from Moravek Biochemicals (Brea, California, USA), FdUMP·Na and FH_4 from Sigma (St Louis, Missouri, USA), and TS (derived from *Lactobacillus casei*) from Biopure (Boston, Massachusetts, USA). All other reagents were the highest available grade.

TS assay

We measured cytosolic FdUMP and [^{3}H]FdUMP binding sites present in TS using a reported method with some modifications [8,9]. In brief, HST-1 cells were seeded into tissue culture dishes (100 mm) at a density of 4×10^6 cells and incubated overnight. Then the cells in 10 dishes were exposed to each schedule of 5-FU. Subsequently, the cells were mechanically detached by a scraper and cells from 10 dishes were collected together. The collected samples were homogenized and sonicated in 2 ml of 50 mmol/l KH_2PO_4 buffer, pH 7.4, containing 20 mmol/l 2-mercaptoethanol (ME), 100 mmol/l NaF and 15 mmol/l cytidylate at 4°C. The cytosol then was prepared by centrifugation and 1 ml of the cytosol mixed with 2 ml of 1 mol/l acetic acid was used for the FdUMP assay. The mixture was centrifuged and the supernatant was lyophilized. The acid extract then was loaded on a DEAE cellulose column (Wako Pure Chemical Industry, Osaka, Japan) to separate FdUMP and 2′-deoxyuridine-5′-monophosphate (dUMP). FdUMP was measured by a competitive ligand-binding assay.

To determine the TS_{total}, 50 μl of the cytosol was added to 50 μl 50 mmol/l Tris containing 100 mmol/l NaF, 15 mmol/l cytidine 5′-monophosphate and 100 mmol/l 2-ME (pH 8.0), and the resulting solution incubated for 3 h at 25°C in order to dissociate FdUMP from the complex. After incubation, 9.5 pmol [^{3}H]FdUMP (0.15 mCi) and 25 μl of a solution containing 2 mmol/l tetrahydrofolic acid, 16 mmol/l sodium ascorbate and 9 mmol/l formaldehyde were added, followed by incubation for 20 min at 25°C. To this solution was added 1 ml of a cold slurry comprising 3.3 g activated charcoal in 100 ml 0.1 mol/l HCl containing 3% dextran and 2% BSA, and the suspension was allowed to stand for at least 40 min in an ice-water bath. The [^{3}H]FdUMP-bound ternary complex was collected in the supernatant by centrifugation, 850 μl of which was transferred to a scintillation vial and mixed with 8 ml Scintisole EX-H, and the radioactivity determined with a liquid scintillation counter. The same procedure was used for purified *L. casei* TS with previously quantitated [^{3}H]FdUMP binding sites as the standard protein. Thus, the sum of [^{3}H]FdUMP binding sites in samples, TS_{total}, was calculated from the standard curve based on *L. casei* TS. TS_{free} was determined in the same manner as TS_{total}, except without incubation for 3 h at 25°C. Further, the inhibition rate of TS activity with FdUMP was calculated using the formula $[1-(TS_{free}/TS_{total})] \times 100$.

Measurement of 5-FU incorporated into RNA

F-RNA was assayed using gas chromatography–mass spectrometry (GC-MS) with some modifications as described previously [10]. In brief, collected treated samples were homogenized in 2 ml water, mixed with 5 ml cold 5% trichloroacetic acid (TCA) and centrifuged. The resulting precipitate was washed twice by mixing with each of 5 ml cold 5% TCA, 70% ethanol, 95% ethanol and ethanol:diethylether (3 : 1) solvent, followed by centrifugation and discarding the supernatant. The final precipitate was dissolved in 0.3 mol/l KOH and incubated overnight at 37°C to hydrolyze RNA to mononucleotide. After neutralizing with $HClO_4$ and desalting, a portion of the mononucleotide solution was used for the determination of the concentration of RNA in terms of the color reaction between mononucleotide and orcinol and standard RNA from bakers yeast. To 1.4 ml of the residual mononucleotide solution was added 100 μl of 1 μg/ml [$^{15}N_2$]5-FU as internal standard, and this solution was mixed with an equivalent volume of 12 mol/l HCl and hydrolyzed in a closed tube for 20 h at 100°C. After cooling and washing the reaction solution with $CHCl_3$, the solution was evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 1 mol/l phosphate buffer (pH 4.0) and extracted with ethyl acetate. The extract was purified by preparative silica gel column chromatography. The residue of the fraction containing 5-FU and internal standard was dissolved in 30 μl acetonitrile, and reacted with 10 μl ditrifluorobenzyl bromide in the presence of triethylamine for 20 min at room temperature. Then 50 μl ethyl acetate followed by 450 μl *n*-hexane was added to the reaction mixture, and a precipitate formed in the solution. After centrifugation of the solution, the supernatant was transferred to a new tube. The solvent was removed under a stream of nitrogen and the residue was reconstituted with 200 μl *n*-hexane as the sample solution for GC-MS.

GC-MS analysis was carried out with a system consisting of a Hewlett Packard 5890 (Hewlett Packard, Tokyo, Japan) gas chromatograph and a JEOL Automass JMS-AM150 (JEOL, Tokyo, Japan) mass spectrometer. To the gas chromatograph was connected a DB-1 fused silica capillary column, 30 m × 0.25 mm internal diameter (J & W Scientific, Folsom, California, USA). The GC conditions were as follows: the carrier gas was helium at a

flow rate of 1.0 ml/min at the outlet of the column, the injector temperature was 250°C, the interface temperature was 250°C and the oven temperature was maintained at 100°C for 1 min, then programmed to increase at 20°C/min to 300°C and maintained at 300°C for 10 min. An aliquot (1 µl) of the sample solution was injected into the GC-MS in splitless mode. The mass spectrometer was operated under negative-ion chemical ionization mode. Isobutane was introduced into the ion source at about 0.5 Torr as reagent gas. The source temperature was 150°C and the ionization energy was 150 eV. 5-FU and internal standard were monitored with ions of *m/z* 355 and 357, respectively. The analysis was based on an established procedure. The apparatus for analysis was regularly checked with quality control samples. We calculated the mean ± SD values from the analytical data for each item to be used as the supplemental data for validation.

Cell survival assay

To avoid the density-dependent inhibition of cell growth, an appropriate number of cells (1×10^4) was seeded into 60-mm culture dishes (Falcon 3002; Oxnard, California, USA) such that control cultures did not reach confluence at the time of harvest. The cells were allowed to attach to the bottom overnight. In the low AUC, cells were exposed to 1 µg/ml of 5-FU for 24 h and 24 µg/ml of 5-FU for 1 h, while in the high AUC, cells were exposed to 5 µg/ml of 5-FU for 24 h and 120 µg/ml of 5-FU for 1 h. After drug treatment, the medium was replaced with fresh medium and the cells were cultured for 10 days with medium change every 3 days. The control dishes were cultured in the medium without drug. The cell number was counted using a Coulter counter (Model ID; Hialeah, Florida, USA). The percentage of survival was calculated by dividing the number of cells in the drug-treated culture by the number of cells in the culture not exposed to drug. The data include results from three separate experiments.

Statistical analysis

For statistical analysis, Student's *t*-test was used to compare the differences in F-RNA contents, FdUMP levels, TS inhibition rate and growth-inhibition rate between the two different schedules of 5-FU. Differences were considered statistically significant at $P < 0.05$.

Results

Detection of RNA incorporation

To simulate the bolus and infusional treatments of 5-FU, we treated HST-1 cells either with a high dose of 5-FU for a short-term or a low dose of 5-FU for a long-term, so that the AUC became equivalent between both treatment schedules. Exposure to 5-FU for 24 h caused an IC_{50} at the concentration of 1.0 µg/ml. Therefore, in the low AUC, cells were treated with either 1 µg/ml for 24 h or 24 µg/ml for 1 h, while in the high AUC, cells were treated with either 5 µg/ml for 24 h or 120 µg/ml for 1 h. When the cells were treated with a low AUC, F-RNA contents in the cells treated with 1 µg/ml for 24 h exposure and 24 µg/ml for 1 h exposure were 264 and 67.1 ng/mg RNA, respectively. When the cells were treated with a high AUC, F-RNA contents in the cells treated with 5 µg/ml for 24 h exposure and 120 µg/ml for 1 h exposure were 759 ± 62.9 and 375 ± 62.9 ng/mg RNA, respectively. F-RNA contents increased as AUC of 5-FU increased and were 2–4 times significantly higher in the cells treated with low-dose, long-term exposure than those treated with high-dose, short-term exposure, indicating that the levels of F-RNA is AUC and schedule dependent (Table 1).

Measurements of FdUMP, TS activity and TS inhibition rate

Although the amounts of FdUMP, total TS and free TS were not detected in the cells treated with a low AUC, except total TS in a long-term schedule, these parameters were available in the cells treated with a high AUC. As shown in Table 1, FdUMP levels in the cells treated with 5 µg/ml for 24 h exposure and 120 µg/ml for 1 h exposure were 20.1 ± 19.4 and 4.56 ± 3.56 ng/mg RNA, respectively. FdUMP levels of long-term exposure appeared higher than those of short-term exposure. There was no statistical difference, however. The total TS levels in the cells treated with 5 µg/ml for 24 h exposure and 120 µg/ml for 1 h exposure were 4.68 ± 4.02 and 3.08 ± 4.38 pmol/g, respectively. Free TS levels in the cells treated with 5 µg/ml for 24 h exposure and 120 µg/ml for 1 h exposure were 0.96 ± 0.74 and 0.95 ± 0.64 pmol/g, respectively. Thus, TS inhibition rates in the cells treated with 5 µg/ml for 24 h exposure and 120 µg/ml for 1 h exposure were 75.4 ± 14.4 and 74.9 ± 14.9%, respectively. Consequently, FdUMP levels, TS activity and TS inhibition rate all increased as the AUC of 5-FU increased, but no significant differences were found between low-dose, long-term exposure and high-dose, short-term exposure, suggesting that FdUMP levels, TS activity and TS inhibition rate are AUC dependent, but not schedule dependent.

Cytotoxicity assay of 5-FU

When the cells were treated with a low AUC, the inhibition rates of cell growth after 10 days in the cells treated with 1 µg/ml for 24 h exposure and 24 µg/ml for 1 h exposure were 75 ± 7 and 43 ± 16%, respectively. When the cells were treated with a high AUC, the inhibition rates in the cells treated with 5 µg/ml for 24 h exposure and 120 µg/ml for 1 h exposure were 96 ± 3 and 72 ± 5%, respectively. These data indicated that the activity of 5-FU increased as the AUC of 5-FU increased and were 1.3–1.7 times significantly more cytotoxic in the cells treated with low-dose, long-term exposure than those treated with high-dose, short-term exposure, indicating that the cytotoxicity of 5-FU is AUC and schedule dependent (Table 2).

Table 1 F-RNA, FdUMP content and TS activity in HST-1 cells treated with two different schedules of 5-FU

Dose intensity	Treatment schedule	F-RNA (ng/mg RNA)		FdUMP (ng/mg RNA)		TS activity: TS total (pmol/g)		TS activity: TS free (pmol/g)		TS activity: Inhibition rate (%)	
Low AUC[a]	1 μg/ml (24 h exposure)	264	[3c]	ND[b]		2.4		ND		-	
	24 μg/ml (1 h exposure)	67.1		ND		ND		ND		-	
High AUC	5 μg/ml (24 h exposure)	759 ± 62.9	[3c]	20.1 ± 19.4	[NS]	4.68 ± 4.02	[NS]	0.96 ± 0.74	[NS]	75.4 ± 14.4	[NS]
	120 μg/ml (1 h exposure)	375 ± 62.9		4.56 ± 3.56		3.08 ± 4.38		0.95 ± 0.64		74.9 ± 14.9	

In the low AUC, cells were treated with either 1 μg/ml for 24 h or 24 μg/ml for 1 h, while in the high AUC, cells were treated with 5 μg/ml for 24 h or 120 μg/ml for 1 h. The values are means ± SD of three independent experiments. NS: not significant.
[a]The experiment was done once at this concentration, because the parameters other than F-RNA could not be detected.
[b]ND: not detected.
[c]$P<0.05$ by Student's *t*-test.

Table 2 Cytotoxicity of 5-FU in HST-1 cells treated with two different schedules

Dose intensity	Treatment schedule	Actual no. of cells/dish (× 104)		Survival fraction		Growth inhibition rate (%)	
	No treatment	50.4 ± 3.82		1		0	
Low AUC	1 μg/ml (24 h exposure)	12.5 ± 3.54	[a]	0.25 ± 0.07	[a]	75 ± 7	[a]
	24 μg/ml (1 h exposure)	28.7 ± 8.08		0.57 ± 0.16		43 ± 16	
High AUC	5 μg/ml (24 h exposure)	2.0 ± 1.41	[b]	0.04 ± 0.03	[b]	96 ± 3	[b]
	120 μg/ml (1 h exposure)	14.3 ± 2.31		0.28 ± 0.05		72 ± 5	

In the low AUC, cells were treated with 1 μg/ml for 24 h and 24 μg/ml for 1 h, while in the high AUC, cells were treated with 5 μg/ml for 24 h and 120 μg/ml for 1 h. The values are means ± SD of three independent experiments.
[a]$P<0.05$ by Student's *t*-test.
[b]$P<0.01$ by Student's *t*-test.

Discussion

Although continuous infusion of 5-FU has been clinically demonstrated to be superior to bolus administration [2–4], the mechanistic difference between the treatments has yet to be determined. Therefore, by simulating bolus and continuous infusion *in vitro*, we investigated the mechanisms of growth inhibition by directly measuring the parameters including 5-FU incorporated into RNA (F-RNA), FdUMP and TS inhibition. We found that both F-RNA content and cytotoxicity of 5-FU increased with elevation of the AUC of 5-FU and that the cells treated with a low dose of 5-FU for a long-term exposure exhibited 2–4 times significantly more F-RNA content and 1.3–1.7 times significantly higher cytotoxicity, respectively, than those administered with the high-dose, short-term exposure. These data, together with the fact that the extent of 5-FU-mediated TS inhibition did not differ significantly between the two different schedules, suggest that increases of 5-FU activity on continuous versus short-term exposure might be explained by the increase of F-RNA content. This increase of F-RNA might be conceivable because 5-FU is a cell cycle-specific drug that exhibits its anti-tumor activity through incorporation into DNA and RNA during both G_1 and S phases. Upon short-term exposure, substantial numbers of cells will not enter the G_1/S phase, thus evading 5-FU-induced DNA and RNA damage [11].

It has been widely accepted that F-RNA is an important element of 5-FU cytotoxicity since there is a good correlation between the cytotoxicity and incorporation of 5-FU into RNA [12]. Decreased drug incorporation into RNA has been observed in 5-FU-resistant cells [13,14]. In addition, it has been suggested that measurement of F-RNA levels together with the determination of 5-FU concentration and TS inhibition rate should be considered as good parameters for the evaluation of anti-tumor efficacy of 5-FU and its analogs both in experimental and in clinical settings [15]. 5-FU has been shown to be extensively incorporated into both nuclear and cytoplasmic RNA species, interfering with normal RNA processing and function [12]. Nuclear 'run-on' transcription analysis revealed that 5-FU inhibited RNA transcription [16]. 5-FU can be incorporated into RNA, which will lead to non-DNA damage-directed effects such as disturbances at the transcriptional and post-transcriptional level [17]. The mechanism whereby 5-FU modulates the expression of mRNA transcripts remains unclear. Misincorporation of 5-FU into RNA may potentially affect many RNA processes important for mRNA function. It is quite possible that the RNA-directed cytotoxicity of 5-FU is due to the combination of many actions against RNA functions which simply overwhelm the cells, resulting in death [12].

It has been shown *in vitro* that short-term treatment with 5-FU produced resistance via decreased incorporation of FUTP into RNA, while repeated prolonged exposure to 5-FU produced resistance via rapid recovery from TS activity [5], suggesting that 5-FU administered in bolus

and continuous fashions may preferentially cause RNA- and DNA-directed (inhibition of TS) cytotoxicity, respectively. In addition, TS inhibition in cancer tissues has been shown to be significantly higher in patients treated with infusional 5-FU than those treated with the bolus administration [6]. Contrary to these studies, we have clearly shown here that long-term treatment did not increase the TS inhibition rate as compared to short-term treatment. Rather, long-term treatment enhanced the incorporation of 5-FU into RNA. Although FdUMP levels, total TS, free TS and TS inhibition rate all increased with elevation of the AUC of 5-FU, no significant differences in these parameters could be observed between low-dose, long-term and high-dose, short-term exposures, suggesting that these parameters are AUC dependent, but not schedule dependent. The mechanism of the lack of differences in these parameters between low-dose, long-term and high-dose, short-term treatments remains to be fully elucidated. The kinetics of 5-FU in murine tumor models *in vivo* and human carcinoma cells *in vitro* has shown that FdUMP levels were highest at the earliest 0.5 h, with rapid losses within 1 h after treatments [13,18,19]. In our study, the cells were treated with a low dose for a 1-h exposure and a high dose for a 24-h exposure. By catabolism and binding to free TS, newly produced FdUMP may be eliminated rapidly, resulting in no significant differences in FdUMP levels and subsequent TS inhibition rates between these schedules.

In conclusion, low-dose, long-term treatment of 5-FU exhibited higher growth-inhibition rates as well as higher levels of F-RNA than the high-dose, short-term treatment. Because the extent of 5-FU-mediated TS inhibition did not differ significantly between the two different schedules, superior activity of 5-FU administered as a low-dose, long-term exposure over high-dose, short-term exposure could be explained by more 5-FU incorporated into RNA during long-term exposure. Our data provide a strategic rationale for the clinical advantage of continuous infusion of 5-FU over bolus administration.

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In-vitro schedule-dependent interaction between oxaliplatin and 5-fluorouracil in human gastric cancer cell lines

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In order to define the most effective combination schedule of oxaliplatin (L-OHP) and 5-fluorouracil (5-FU), we investigated the in vitro interaction between these drugs in a panel of four human gastric adenocarcinoma cell lines (MKN-1, NUGC-3, NUGC-5 and AZ-521). Cytotoxic activity was determined by the WST-1 assay. Different schedules of the two drugs were compared and evaluated for synergism, additivity or antagonism with a quantitative method based on the median-effect principle of Chou and Talalay. Cell cycle perturbation and apoptosis were evaluated by flow cytometry. Simultaneous and sequential treatments of L-OHP followed by 5-FU exhibited synergistic effects in all four cell lines, whereas the reverse sequence yielded a clear antagonism. 5-FU exclusively arrested cells at the G_0/G_1 phase, and L-OHP at the G_0/G_1 and G_2/M phases. Apoptosis was most prominent when cells were treated simultaneously or in a sequence of L-OHP followed by 5-FU, producing apoptosis in the majority of treated cells (55.5–61.5%). In contrast, the reverse sequence yielded only 20% induction of apoptosis, the rate being not significantly different from those induced by each drug singly. Moreover, this sequence dependence was further confirmed by the experiment which compared the total number of NUGC-3 cells 7 days after these combination schedules. These findings suggest that the interaction of 5-FU and L-OHP could be highly schedule dependent, with the most efficacious interaction observed in simultaneous combination and that 5-FU followed by L-OHP would not be recommended in clinical trials for patients with advanced gastric cancer. *Anti-Cancer Drugs* 17:445–453

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Introduction

Gastric cancer is the second most common cause of cancer death worldwide, with most cases being diagnosed at the stage of advanced disease [1,2]. The prognosis for this disease is extremely poor, with a median survival time ranging between 6 and 8 months [3,4]. Only 10% of patients with advanced gastric cancer can survive 2 years with chemotherapy [5]. Although gastric cancer appears to be a chemotherapy-sensitive disease, there have been no standard chemotherapeutic regimens for this incurable disease. Therefore, it is of paramount importance to develop the most beneficial chemotherapeutic regimens that improve survival of patients.

Oxaliplatin (*trans*-l-1,2-diaminocyclohexane oxalato platinum II, L-OHP) is a third-generation platinum compound that acts as an alkylating agent, inhibiting DNA replication by forming adducts between two adjacent guanines or guanine and adenine [6]. L-OHP has been demonstrated to exhibit anti-tumor activity against several cell lines with acquired cisplatin resistance as well as clinical tumors that are intrinsically resistant to cisplatin and carboplatin [7,8]. Phase II studies of single-agent L-OHP have shown activity in colorectal [9], ovarian [10], breast [11] and non-small cell lung cancers [12]. Moreover, L-OHP has been shown recently to be effective against gastric cancer in several phase II studies [13,14]. L-OHP has a different toxicity profile from that of cisplatin, with mild nausea/vomiting and, in contrast to carboplatin, mild to moderate hematological toxicity. The dose-limiting toxicity of L-OHP is a dose-dependent and reversible peripheral neuropathy [15].

Currently, the combination of 5-fluoruracil (5-FU)/leucovorin and L-OHP is regarded as a standard regimen for patients with advanced colorectal cancer. This combination was administered with the sequence L-OHP/leucovorin followed by 5-FU [16]. However, the optimal combination schedule of 5-FU and L-OHP still remains unclear. Fischel *et al.* demonstrated that the combination of L-OHP and 5-FU is synergistic whatever the tested schedules using four human colorectal cancer cell lines [17]. Since this combination has been shown to be effective against patients with advanced gastric cancer [14,18–20], we investigated the schedule-dependent interaction between 5-FU and L-OHP using a panel of

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human gastric cancer cell lines. We have found that the interaction of 5-FU and L-OHP could be highly schedule dependent.

Materials and methods

Cell lines and culture

Four human gastric cancer cell lines (AZ-521, MKN-1, NUGC-3 and NUGC-5) were purchased from the Japanese Cell Resource Bank (Tokyo, Japan). The cells were maintained in DMEM (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated FBS (Gibco, Grand Island, New York, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified incubator under an atmosphere containing 5% CO_2.

Drugs

5-FU and L-OHP were kindly provided by Kyowa Hakko (Tokyo, Japan) and Yakult (Tokyo, Japan), respectively. Stock solutions of these drugs were prepared in sterile distilled water. Immediately before their use, 5-FU and L-OHP were dissolved in culture medium.

Evaluation of cytotoxicity

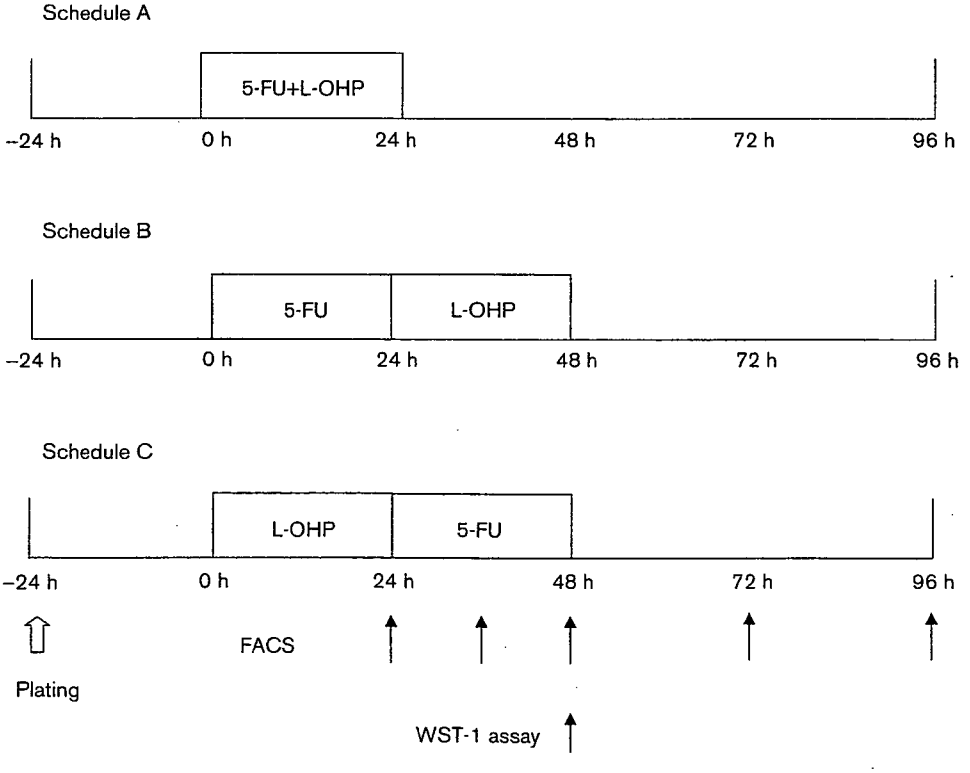
Cytotoxic activity was measured by the WST-1 assay (Wako Chemicals, Osaka, Japan), following the manufacturer's instructions [21]. The WST-1 assay is a colorimetric method in which the intensity of the dye is proportional to the number of viable cells. Briefly, exponentially growing cells were plated into 96-well microplates at a density of 3000 cells/well in a volume of 100 μl/well and incubated for 24 h for sufficient cell growth. The cells were then treated with graded concentrations of 5-FU or L-OHP alone for 24 h, or in simultaneous or sequential fashion, as shown in Fig. 1.

After treatment, the cells were washed twice with PBS, and cultured in drug-free medium for an additional 21 h. Then, 10 μl of WST-1 solution was added into each well and the plates were incubated at 37°C for 3 h. Absorbance values at 450 and 620 nm were measured using a Delta Soft Elisa analysis program for Macintosh computers interfaced with a microplate reader (Immuno-Mini NJ-2300; Bio-Tek, Winooski, Vermont, USA). Wells containing cells untreated with drugs were used as controls. Each experiment was performed using six replicate wells for each drug concentration and carried out independently at least 3 times. The IC_{50} values were defined as the concentrations that inhibited 50% of cell growth.

Functional interactions between drugs

The combined drug effects were evaluated by using the Chou and Talalay analysis based on the median-effect

Fig. 1



Description of the three combination schedules. Closed arrows indicate the harvest of samples for FACS analysis and WST-1 assay.

principle [22]. This method involves plotting dose–effect curves for each drug and for multiply diluted, fixed-ratio combinations by using the median-effect equation: $f_a/f_u = (D/D_m)^m$, where D is the dose, D_m is the dose required for 50% effect (e.g. 50% inhibition of cell growth), f_a is the fraction affected by dose D (e.g. 0.9 if cell growth is inhibited by 90%), f_u is the unaffected fraction (therefore $f_a = 1 - f_u$) and m is a coefficient of the sigmoidicity of the dose–effect curve. Based on the slope of the dose–effect curves, it can be determined whether the drugs have mutually non-exclusive effects (e.g. independent or interactive mode of action).

The combination index (CI) is then determined by the equation: $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + \alpha(D)_1(D)_2/(D_x)_1(D_x)_2$, where $(D_x)_1$ is the dose of drug 1 required to produce x percent effect alone and $(D)_1$ is the dose of drug 1 required to produce the same x percent effect in combination with $(D)_2$. If the mode of action of the drugs is mutually exclusive or non-exclusive, then α is 0 or 1, respectively. CI values were calculated by solving the equation for different values of f_a (i.e. different degrees of inhibition of cell growth). CI values below 1 indicate synergy, values equal to 1 indicate additive effects and values above 1 indicate antagonism.

Data analysis was performed automatically using the CalcuSyn software program (Biosoft, Cambridge, UK). The dose–effect relationships for the drugs tested alone or in combinations were subjected to the median-effect plot in order to determine their relative potency (IC_{50}), shape (m) and conformity (r) in each selected cell line. As defined previously, the IC_{50} and m values were used for calculating synergism or antagonism based on the CI equation.

Cell cycle analysis

AZ-521 cells were seeded at a density of 3×10^5 per 100-mm dish (3003; Falcon, Oxnard, California, USA). Then the cells were treated with 5-FU or L-OHP singly, or concurrent or sequential combinations, as shown in Fig. 1. After medium change, the cultures were continued until cell cycle analyses 24, 36, 48, 72 and 96 h after the beginning of treatment. The cells were harvested by collecting floating and trypsinized adherent cells, and fixed in 70% ethanol in PBS for at least 30 min on ice. After removal of ethanol by centrifugation, cells were washed with ice-cold PBS and then incubated in PBS containing 45 μg/ml propidium iodide (PI) and 500 μg/ml ribonuclease A (Sigma, St Louis, Missouri, USA) for 30 min on ice in the dark. Cell cycle analysis was performed on a Becton Dickinson FACSCalibur flow cytometer using the CellQuest and ModFit 3.0 software packages (Becton Dickinson, San Jose, California, USA). The percentages of apoptotic populations were determined by measuring the sub-G_1 phase after collecting floating and trypsinized adherent cells at various times following drug exposure. Results were obtained from three separate experiments performed in duplicate.

Statistical analysis

Statistical significance between these combination treatments was determined by Student's t-test. Significant differences were considered at $P < 0.05$.

Results

Single-agent experiments

The cytotoxic activities of 5-FU and L-OHP were tested individually on the four tumor cell lines. The cells were exposed to each drug for 24 h. The IC_{50} are summarized in Table 1. For 5-FU, the IC_{50} ranged from 16.1 μmol/l for AZ-521 cells to 344 μmol/l for MKN-1 cells. NUGC-3 and NUGC-5 cells showed IC_{50} values of 115 and 129 μmol/l, respectively. For L-OHP, AZ-521 cells were the most sensitive to L-OHP (3.27 μmol/l) among the four tumor cell lines and MKN-1 cells were the least sensitive (15.4 μmol/l). The IC_{50} values of NUGC-3 and NUGC-5 cells were 13.3 and 14.1 μM, respectively.

Median-effect analysis of 5-FU and L-OHP combination *in vitro*

5-FU and L-OHP were tested in different combinations to define the most effective schedule. Three different schedules (simultaneous and sequential drug exposures) were tested as shown in Fig. 1 and the exposure duration to each drug was 24 h. In MKN-1 cells, both simultaneous treatment and the sequence L-OHP followed by 5-FU showed synergistic effects (Fig. 2a and c), while the sequence 5-FU followed by L-OHP exhibited an antagonistic effect at almost all ranges of the cell kill fractions (Fig. 2b). In NUGC-3 cells, simultaneous treatment and the sequence L-OHP followed by 5-FU also showed a remarkable synergism at all cell kill fractions (Fig. 3a and c). In contrast, the reverse sequence (5-FU followed by L-OHP) demonstrated a clear antagonism at all cell kill fractions (Fig. 3b). In NUGC-5 cells, simultaneous treatment and sequence L-OHP followed by 5-FU produced a marked synergism at all ranges of cell kill fractions (Fig. 4a and c), whereas the opposite sequence 5-FU followed by L-OHP produced an antagonism at all cell kill fractions (Fig. 4b). In AZ-521 cells, simultaneous treatment and the sequence L-OHP followed by 5-FU yielded a synergism (Fig. 5a and c).

Table 1 IC_{50} values of 5-FU and L-OHP in four gastric cancer cell lines

Drug	MKN1	NUGC-3	NUGC-5	AZ-521
5-FU (μmol/l)	344 ± 43.8	115 ± 70.0	129 ± 45.4	16.1 ± 0.77
Oxaliplatin (μmol/l)	15.4 ± 9.06	13.3 ± 7.3	14.1 ± 11.6	3.27 ± 0.76

Cells were treated with various concentrations of 5-FU for 24 h or L-OHP for 24 h, and assayed for cytotoxicity as described in Materials and methods. The values are the means ± SD of three independent experiments.