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Abstract

Conventional therapy for colorectal carcinoma using 5-fluorouracil (5-FU) has shown limited antitumor action. The purpose of our study was to investigate synergistic antitumor effects of the streptococcal preparation of OK-432 and 5-FU, and to elucidate the mechanisms of interaction between the 2 agents in mice. Biochemical modulation of OK-432 and 5-FU were determined in vivo against colon-26 carcinoma. The concentration of 5-FU and its metabolites, and the activity of thymidylate synthase and thymidine kinase, respectively, were measured using cytosolic extracts of the tumors. Combination treatment with OK-432 produced a significant increase in intratumor 5-FU and 5-FU in RNA (F-RNA) concentrations, increased the thymidylate synthetase inhibition rate, and decreased thymidine kinase activity, as compared with the results observed in the control mice. These additive antitumor effects are obtained by use of the 2 agents; the mechanism of action is considered to be the suppression of both the de novo and the salvage pathway for DNA synthesis, along with the suppression of RNA synthesis.

KEYWORDS: OK-432, 5-FU, biochemical modulation

Biochemical Modulation of 5-Fluorouracil with a Streptococcal Preparation, OK-432, against Murine Colon-26 Carcinoma

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Conventional therapy for colorectal carcinoma using 5-fluorouracil (5-FU) has shown limited antitumor action. The purpose of our study was to investigate synergistic antitumor effects of the streptococcal preparation of OK-432 and 5-FU, and to elucidate the mechanisms of interaction between the 2 agents in mice. Biochemical modulation of OK-432 and 5-FU were determined in vivo against colon-26 carcinoma. The concentration of 5-FU and its metabolites, and the activity of thymidylate synthase and thymidine kinase, respectively, were measured using cytosolic extracts of the tumors. Combination treatment with OK-432 produced a significant increase in intratumor 5-FU and 5-FU in RNA (F-RNA) concentrations, increased the thymidylate synthetase inhibition rate, and decreased thymidine kinase activity, as compared with the results observed in the control mice. These additive antitumor effects are obtained by use of the 2 agents; the mechanism of action is considered to be the suppression of both the de novo and the salvage pathway for DNA synthesis, along with the suppression of RNA synthesis.

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C urrent approaches to the development of new therapies for patients with gastrointestinal carcinoma have taken 2 paths: the continued search for new pharmacologic agents and the development of innovative approaches such as the use of monoclonal antibodies, vaccines, and other biologic agents. In addition, investigations continue to focus on the biochemical modulation of 5-fluorouracil (5-FU), an agent that has been used for over 30 years and which remains the most active single agent yet developed for treating patients with gastrointestinal carcinoma. Attempts to biomodulate the activity of 5-FU have focused principally on enhancing its ability to inhibit the target enzyme thymidylate synthase (TS). This enzyme, essential in the de novo synthesis of thymidylate, is believed to represent an important chemotherapeutic target for several reasons. First, there is a close association between the intracellular expression of this enzyme and the sensitivity of cells to fluoropyrimidines (1). Second, several clinical investigations have demonstrated an association between the degree of inhibition of TS and a positive clinical outcome in patients with colorectal and breast cancers (2). Third, leucovorine (LV), a commercially available reduced folate, specifically enhances the ability of 5-FU to inhibit TS activity. For the last 10 years, clinical investigations using LV have demonstrated an approximate doubling in the response rate of patients with advanced colorectal carcinoma treated with 5-FU (3). Finally, preclinical studies identified the existence of synergistic effects when interferons (IFNs) were coadministered with 5-FU cultures of adenocarcinoma 38 and HL-60 cell lines; however, IFNs are inactive as solitary agents (4, 5). Several pilot clinical trials demonstrated that biochemical modulation of 5-FU with IFNs would be feasible and effective in cases advanced colorectal cancer (6, 7).

OK-432 is a penicillin-inactivated and lyophilized preparation of the low-virulence strain Su of Streptococcus pyogenes; its immunomodulatory and potential therapeutic properties as a biological response modifer have been previously described (8). Clinically, this preparation has been extensively used in Japan as an adjuvant treatment for patients with lung cancer (9), esophageal cancer (10), gastrointestinal cancer (11) and peritoneal car-

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cinomatosis (12). Immunotherapy with OK-432 has led to improved cancer survival rates when used concomitantly with chemotherapy, such as per oral administration of 5-FU prodrugs. However, the precise mechanism of its anti-tumor action, or of chemotherapy combined with OK-432, has not been fully elucidated. The efficacy of OK-432 has been variably attributed to T cell and natural killer cell activation, macrophage-mediated cytotoxicity, neutrophil activation, and multicytokine production (13–16). It has been shown that OK-432 is a multicytokine inducer with interleukin-2 (IL-2), and thus plays a major role in natural killer cell activation and interferon-γ (IFN-γ) production (17). In addition, OK-432 has recently been reported to be a potent inducer of an IL-12 and T Helper Cell 1-dominant state (18). The synergistic antitumor effects and prolonged cancer survival rates of chemotherapy with OK-432 have been attributed to the immune activation of the host. On the other hand, it is speculated that another mechanism of the synergistic antitumor effects of OK-432 and 5-FU is the biochemical modulation of 5-FU. The purpose of the present study is to investigate the mechanisms of interaction between these 2 agents in the murine colon cell carcinoma cell line.

Materials and Methods

Reagents. OK-432 (Picibanil; Chugai Pharmaceutical Co., Tokyo, Japan) is a lyophilized preparation of group A streptococcus, type 3, Su strain, inactivated with penicillin G. The concentration of OK-432 is expressed in units designated as Klinische Einheit (KE; clinical unit). One KE of OK-432 equals 0.1 mg dried streptococci. Commercially available 5-FU was purchased from Kyowa Hakko Kogyo (Tokyo, Japan).

Animals. CDF1 mice were obtained from Japan SLC (Hamamatsu, Japan). Male mice, 5–6 weeks old, were used in all experiments. Mice were maintained in a pathogen-free barrier containment facility on a regulated 12-h light/dark cycle and were acclimatized for at least 1 week before they were used in any of the experiments.

Cells. Colon 26 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO_2 at 37 °C.

Inoculation of tumor. Subconfluent cells were treated with 0.05% trypsin and 0.02% EDTA to obtain a single-cell suspension and were resuspended at a concentration of 5×10^6 cells/ml in PBS. Then 1×10^6 cells/0.2 ml were inoculated subcutaneously into the right

lower back of CDF1 mice (day 0).

In vivo procedure. OK-432 was diluted in PBS at a concentration of 20 KE/ml and injected intratumorally at a volume of 0.05 ml (0.1, 1.0 KE/mouse) into the mice. In the control group, an equal amount of PBS was injected. A single dose of 5-FU 40 mg/kg was given to the mice intraperitoneally (i.p.).

Experimental design. The mice subcutaneously inoculated with colon 26 tumor cells were then randomized into 4 groups (8 per group) and groups (i) and (ii) were given intratumoral PBS/OK-432 injections on day 14, 16, 18, 20, 22, and 24, followed by 5-FU i.p. administration on day 25 (control/OK-432 1D); groups iii and iv were administered intratumoral PBS/OK-432 injections on day 14, 16, 18, 20, 22, and 24, followed by 5-FU administration on day 27 (control/OK-432 3D) (Fig. 1). Mice were sacrificed by cervical dislocation 15 min after 5-FU administration, and tumors freed of epidermal attachments were immediately frozen in liquid N_2 in polyethylene vials. Data shown are the mean values of a total of 5 replicate experiments. No significant differences in tumor weights or volumes were observed among the groups during the experimental period (data not shown).

5-FU assay. Tissue samples were treated by the method of Masuike et al. (19). Briefly, 5-FU in sample solutions was determined by high-performance liquid chromatography (HPLC). The column used was a silica gel column (Develosil 60–3, 4.6×100 mm). The mobile phase was ethyl acetate: n-hexane: 98% formic acid: water (50:50:0.5:0.3), and the eluate was monitored at 264 nm. The limit of detection of 5-FU was 5 ng/g.

5-fluorodeoxuridine monophosphate (FdUMP) assay. This assay was done essentially according to the method of Moran et al. (20). The FdUMP assay was based on competition between ^{3}H FdUMP and unlabeled FdUMP for binding to thymidylate synthetase (TS). The assay was performed at 25 °C and the tube contained the following reactants in a final volume of 275 μl: (1) 8.7 nmol of CH_2-FH$_4$ 25 μl; (2) 0.6 pmol of ^{3}H FdUMP ((3H) FdUMP (22 mCi. mmol)) 50 μl; (3) a standard or unknown quantity of FdUMP 100 μl; and (4) 0.15 pmol of TS (0.3 mol of FdUMP binding sites, Lactobacillus casei) 100 μl. The tubes were incubated at 25 °C for 4 h. A charcoal suspension (1 ml) was added, and the tubes were mixed and centrifuged at $4,400 \times g$ for 20 min. Then, 900 μl of clear supernatant was placed in 8 ml scintisol EX-H

Exp 1.

Control   PBS i.t.   5-FU i.p.

↓ ↓ ↓ ↓ ↓ ↓   ↓

14 16 18 20 22 24   25

OK-432-1D   OK432 i.t.   5-FU i.p.

↓ ↓ ↓ ↓ ↓ ↓   ↓

14 16 18 20 22 24   25

Exp 2.

Control   PBS i.t.   5-FU i.p.

↓ ↓ ↓ ↓ ↓ ↓   ↓

14 16 18 20 22 24   25 27

OK-432-3D   OK432 i.t.   5-FU i.p.

↓ ↓ ↓ ↓ ↓ ↓   ↓

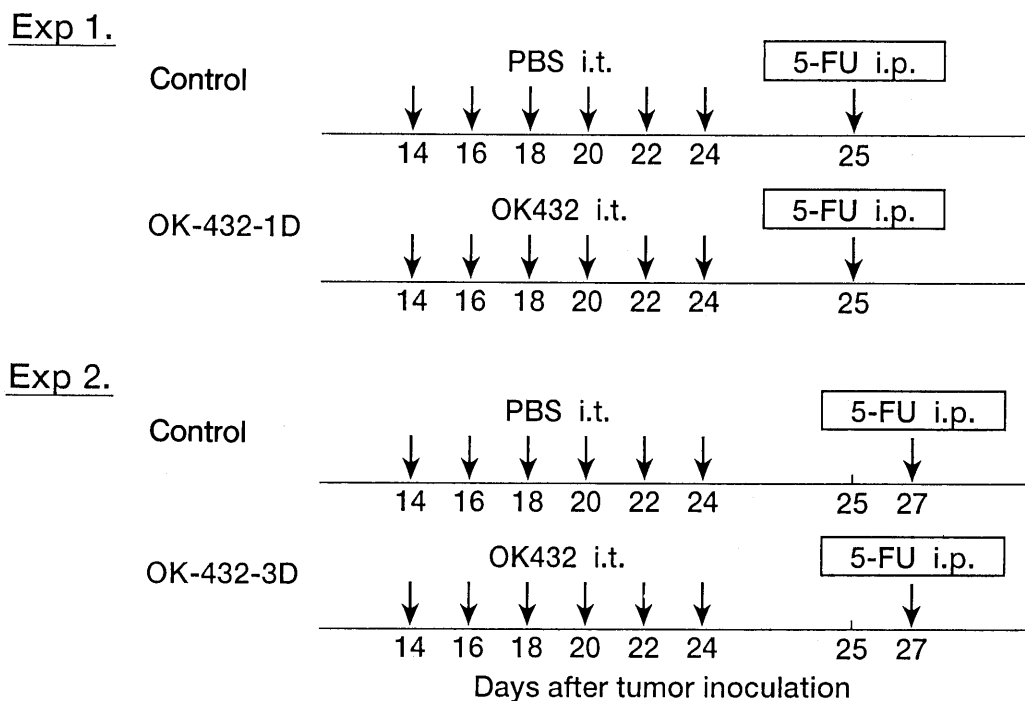14 16 18 20 22 24   25 27

Days after tumor inoculation

Fig. I   Schema of experimental design.
5-FU, 5-fluorouracil; i.p., intraperitoneal; i.t., intratumor administration; PBS, phosphate butter solution.

(Dojin Laboratories, Kumamoto, Japan), and the bound ^{3}H-FdUMP was determined by counting in a liquid scintillation spectro-photometer. CH_2-FH_4 was prepared by the addition of 50 μl of 1 M ascorbate (pH 6.5), 2.2 μl of 37% (v/v) formaldehyde, 9.5 ml of buffer A (1 ml of 1 M phosphate (pH 7.2), 14 μl of 2-mercaptoethanol (2-ME), 2 ml of solution containing 10 mg of bovine serum albumin (BSA) per ml, and 17 ml of water) to contain 3.1 μmol of L-1-(+)-tetrahydrofolate. The charcoal suspension was prepared by mixing 1.67 g of acid-washed activated charcoal (Sigma, MO, USA) with 100 ml of 0.05 N HCl.

5-FU in RNA (F-RNA) assay.   Tissue samples were homogenized with water and aqueous trichloroacetic acid was added, followed by centrifugation. The obtained precipitates were washed repeatedly with ethanol and ethanol-ethylether, and thus lipids and free 5-FU were removed. The precipitates were hydrolyzed under alkaline conditions. Centrifugation ensued and a mononucleotide dilution of RNA was obtained. The RNA conservative concentration of solutions was determined by the orcinol method.   Then, (1,3-bis-^{15}N) 5-FU as internal standard (IS) was added to solutions, which were hydrol-

yzed with HCl to obtain the nucleic acid base. The nucleic acid, base solutions were washed with chloroform and extracted with ethyl acetate. The extracts were purified by preparative silica gel column chromatography. N-0-bis-(trimethylsilyl)-trifluoro-acetamide (BDSTFA) was added to the purified fraction containing 5-FU and IS to convert it to trimethylsilyl derivatives. An aliquot of the reaction solution was used for gas chromatography-mass spectrometry, and the 5-FU and IS levels were monitored using the ions of m/z 259 and m/z 261, respectively, as indices. In this manner, the 5-FU concentration in the mononucleotide solution was determined. The 5-FU concentration in RNA (ng/mg RNA) was calculated from the 5-FU concentration (ng/ml) and the RNA conversive concentration (mg/ml) in the mononucleotide solution.

Thymidilate Synthetase assay. ^{3}H-FdUMP was obtained from Moravec Biochemicals (Brea, CA, USA) and TS (from L. casei) was purchased from Biopure. Homogenized buffer contained: 50 mM KH_2PO_4, 20 mM 2 ME, 100 mM NaF, 15 mM MCMP-2Na (pH 7.4). Buffer A consisted of 300 mM NH_4HCO_3, 100 mM 2-ME, 100 mM NaF, and 15 mM CMP-2Na (pH 8.1). Buffer B consisted of 50 mM KH_2PO_4, 20

mM 2-ME, 100 mM NaF, 15 mM CMP-2Na, and 2% BSA (pH 7.4). Buffer C consisted of 1 mM TH$_4$, 16 mM sodium ascorbate, and 9 mM formaldehyde in buffer B. DDC consisted of acid-washed activated charcoal 3.3 g, BSA 0.83 g, high-molecular-weight dextran 0.083 g, and 100 mM 0.1 N HCl. The TS binding assay was done essentially according to the method of Spears et al. (21). The tissues were minced with scissors to 0.2–0.3 g, homogenized, and sonicated in 4 volumes of homogenized buffer cooled in an ice bath. The material was then prepared by centrifugation at 15,000 × g for 60 min at 4 °C and the supernatant (cytosol) was used for the TS assay. The TS total (TS tot) level was assayed by the addition of 50 μl of Buffer A to 50 μl of cytosol and incubating the mixture for 3 h at 25 °C. The TS levels were assayed by the addition of 50 μl of ³H-FdUMP (40,000 dpm/50 μl) and Buffer C 25 μl. The concentration of TSf^{app}, where TSf^{app} is the experimentally determined concentration of ³H-FdUMP-binding sites present at the end of 20 min in the standard TS assay, was determined in parallel by the omission of the preincubation dissociation period and the addition of Buffer A together with ³H-FdUMP and Buffer C. The tubes were incubated at 25 °C for 20 min, then cold DCC 1 ml was added to permit isolation of protein-bound ³H-FdUMP in the supernatant by 400 × g centrifugation for 20 min. Next, 850 μl of the clear supernatant was placed in 8 ml of scintisol EX-H, and the bound ³H-FdUMP was determined by counting in a liquid scintillation spectrophotometer. The results were calculated using L. casei TS as a standard at pH 7.4. About 13% of TS tot dissociated and rapidly reformed a ternary complex containing ³H-FdUMP during the 20-min TS assay incubation period and appeared as TSf. TSf was therefore calculated from the relationship TSf = (TSf^{app}-0.13 TS tot)/0.87. The percentage inhibition of TS (TSIR) was expressed as the following formula: (TS tot-TSf)/TS tot (%).

Thymidine Kinase assay. The thymidine kinase (TK) assay was performed essentially by the method of Hirata et al. (22). Sections of tissue weighing 0.2–0.3 g were homogenized in 5 volumes of 50 mM Tris-HCl buffer (pH 8,0) containing 10 mM 2-ME, 25 mM KCl, and 5 mM MgCl$_2$ at 4 °C. The homogenate was centrifuged at 105,000 × g for 1 h at 4 °C and the supernatant was used as a crude enzyme preparation. The assay mixture (200 μl) consisted of 100 mM MgCl$_2$ in 0.5 M Tris-HCl buffer (pH 8.0), 50 μl; 100 mM ATP (pH 6

-7) in H$_2$O, 50 ml; 120 μM α-glycerophosphate in H$_2$O, 50 μl; 2 mM dThd in H$_2$O; and 10 μCi/ml (2-¹⁴C) dThd in H$_2$O, 25 μl. This mixture was incubated with the enzyme preparation (100 μl) at 37 °C for 30 min. The reaction was then stopped by boiling for 3 min, followed by centrifugation at 15,000 × g for 15 min at 4 °C. After it was centrifuged, the supernatant (25 μl) was spotted onto a 2 cm diameter disc of DESE-cellulose paper. The paper was washed 2 times with 1 mM ammonium formate with H$_2$O to remove unreacted substrate and was then rinsed with 85% ethanol. The paper was dried and added to 8 ml of xylene-based scintillation fluid, then radioactivity was counted by liquid scintillation. The protein concentration of the enzymatic extracts was estimated by the method of Lowry et al. using BSA as the reference standard.

Statistical analysis. The data represent mean ± SEM and were evaluated for statistical significance by an unpaired Student's t test.

Results

(1) Effect of OK-432 on the level of 5-FU

The concentration of 5-FU was significantly higher, in a dose-dependent manner, in the OK-432-3D group, as compared with the control group. This was not the case in the OK-432-1D group.

(2) Effect of OK-432 on the levels of FdUMP and F-RNA

The concentration of F-RNA was significantly higher, in a dose-dependent manner, in the OK-432-3D group, as compared with the control group; this was not the case in the OK-432-1D group (Table 1). In contrast, no significant difference in FdUMP levels was demonstrated between the control and OK-432-1D/3D group (Table 2).

Table 1 Effect of OK-432 on the level of 5-FU in tumor tissue

		OK-432 (KE)	5FU (mg/g)
Exp 1.	Control	0.0	559.5 ± 60.9
	OK-432-1D	0.1	697.8 ± 73.8
		1.0	797.5 ± 144.1
Exp 2.	Control	0.0	2795.3 ± 351.2
	OK-432-3D	0.1	2257.2 ± 301.3
		1.0	4837.8 ± 580.1*

Values are mean ± SEM. *P < 0.05 vs. control.

(3) Effect of OK-432 on the thymidylate synthetase inhibition rate (TSIR)

The level of TS_{tot} in the OK-432-3D group was not changed from that of the control group, whereas the level of TS_f significantly decreased when compared with that of the control group. The OK-432-3D group thus demonstrated a significant increase in the TSIR dose-dependency, as compared with that of the control group. However, the TSIR in the OK-432-1D group remained unchanged from that of the untreated control group (Table 3).

(4) Effect of OK-432 on thymidine kinase (TK) activity in tumorous tissue

TK activity was significantly decreased in a dose-dependent manner in the OK-432-3D group, as compared with that of the control group. In contrast, the OK-432-1D group did not demonstrate a significant influence on the TK activity of colon-26 tumor (Table 4).

Discussion

There are a limited number of chemotherapeutic options available for the treatment of advanced gastro-

Table 2   Effect of OK-432 on the levels of FdUMP and F-RNA in tumor tissue

		OK-432 (KE)	FdUMP (pmol/g)	F-RNA (ng/mg RNA)
Exp I.	Control	0.0	3.5 ± 1.2	97.5 ± 12.4
	OK-432-1D	0.1	2.5 ± 0.6	97.5 ± 6.5
		1.0	3.7 ± 0.5	96.2 ± 12.9
Exp 2.	Control	0.0	7.4 ± 1.5	14.9 ± 8.9
	OK-432-3D	0.1	6.7 ± 0.7	12.2 ± 10.0
		1.0	6.6 ± 1.1	39.7 ± 8.1*

FdUMP, 5-fluorodeoxuridine; F-RNA, 5-fluorouracil in RNA.
Values are mean ± SEM. *$P < 0.05$ vs. control.

Table 3   Effect of OK-432 on the thymidylate synthetase inhibition rate in tumor tissue

		OK-432 (KE)	TS_{tot} (pmol/g)	TS_f (pmol/g)	TSIR (%)
Exp I.	Control	0.0	73.1 ± 2.4	32.1 ± 1.3	55.7 ± 2.4
	OK-432-1D	0.1	64.4 ± 4.9	30.1 ± 2.2	53.0 ± 0.7
		1.0	72.8 ± 4.5	38.0 ± 2.4	47.5 ± 3.3
Exp 2.	Control	0.0	51.9 ± 6.5	22.3 ± 5.7	57.0 ± 7.1
	OK-432-3D	0.1	55.7 ± 9.9	24.6 ± 9.9	65.0 ± 10.0
		1.0	55.3 ± 2.8	12.1 ± 6.0*	78.1 ± 5.3*

TS, thymidylate synthetase; TS_{tot}, total TS; TS_f, free TS; TSIR, TS inhibition rate.
Values are mean ± SEM. *$P < 0.05$ vs. control.

Table 4   Effect of OK-432 on thymidine kinase activity in tumor tissue

		OK-432 (KE)	TK activity (pmol/min/mg protein)	% inhibition
Exp I.	Control	0.0	0.49 ± 0.71	100.0
	OK-432-1D	0.1	0.59 ± 0.21	120.4
		1.0	0.60 ± 0.12	122.4
Exp 2.	Control	0.0	4.36 ± 0.89	100.0
	OK-432-3D	0.1	2.20 ± 0.58	50.5
		1.0	0.76 ± 0.96*	17.4

TK, thymidine kinase.
Values are mean ± SEM. *$P < 0.05$ vs. control.

intestinal cancer. In advanced disease, 5-FU has only limited activity when administered alone (23). Thus, the search for ways to improve the therapeutic efficacy of 5-FU continues. A review of the metabolic pathways of 5-FU (Fig. 2) shows that several determinants are associated with the cytotoxicity and antitumor activity of this agent (24). These determinants include intracellular concentrations of 5-fluorodeoxyuridine monophosphate (FdUMP) and the competing normal metabolite deoxyuridine monophosphate (dUMP) at the level of thymiddylate synthetase (TS), the amount of drug incorporated into cellular RNA and DNA, and the level of reduced folate cofactor 5, 10-methylenetetrahydrofolate. TS, an enzyme centrally located in the metabolic pathways, is responsible for the methylation of deoxyuridylate to thymidylate, which is a necessary substrate for the synthesis and repair of DNA. The TS reaction requires 5, 10-methylenetetrahydrofolate as a one-carbon donor and is potentially inhibited by one of the active anabolites of 5-FU, namely FdUMP. A number of investigators have demonstrated that the stability of the ternary complex between TS, FdUMP and 5, 10-methylenetetrahydrofolate; this complex depends heavily on the concentration of reduced folate (25). FdUMP forms a covalent complex with TS in the presence of

reduced folates, resulting in TS inhibition, and thus cytotoxic effects are enhanced (26). 5-FU is anabolized to fluorouridine monophosphate (FUMP), and is further phosphorylated to fluorouridine triphosphate (FUTP) and incorporated into RNA (F-RNA), resulting in RNA damage. FdUMP is phosphorylated to FdUTP, which can be directly incorporated into DNA after it is damaged.

This paper aimed to describe one of the mechanisms of the enhanced antitumor activity of 5-FU and OK-432 in combination; this has been shown in several clinical trials (9–12), with reference to the concentrations of 5-FU, FdUMP, and F-RNA and the activities of thymidylate synthetase and thymidine kinase in tumor tissue. OK-432 is a multicytokine inducer with IL-2, IL-12, IFN-γ, TNF-α, and IL-1 (13–16), and these cytokines might play a role in 5-FU uptake in tumor tissue, possibly through increased permeability of the vessel. As shown in Tables 1, 2, 3, and 4, no differences between the examined parameters were detected between the control group and the OK-432-injected (OK-432-1D) group in Exp. 1. These results suggest that cytokine production and their subsequent effects in tumor tissue did not suffice to increase 5-FU uptake when administered one day after OK-432 injection. In addition, the concentrations of 5-FU, FdUMP and F-RNA, and the activ-
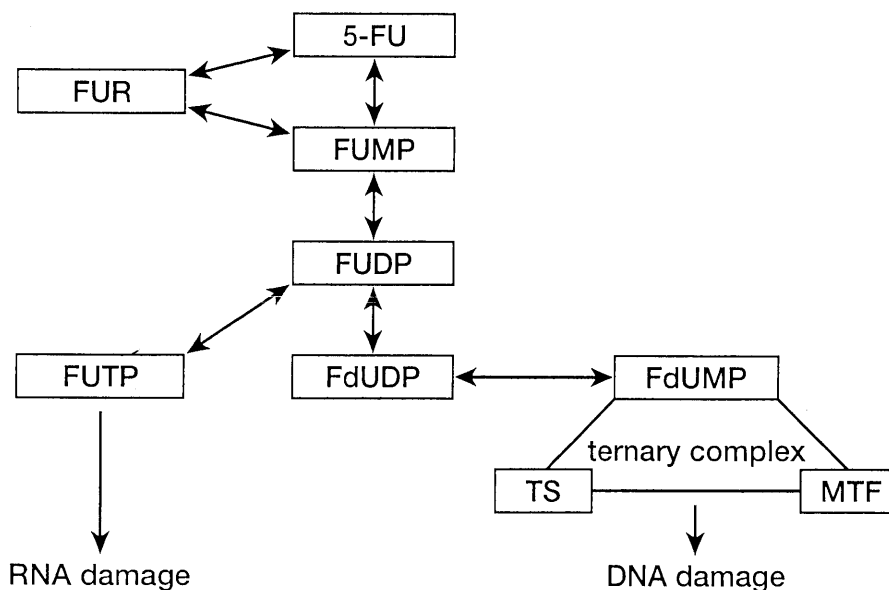

Fig. 2 Schema of metabolic pathway of 5-FU.
5-FU, 5-fluorouracil; FUR, fluorouridine; FUdR, fluorodeoxyuridine; FUMP, fluorouridine monophosphate; FUDP, fluorouridine diphosphate; FUTP, fluorouridine triphosphate; FdUMP; fluorodeoxyuridine monophosphate; MTF, 5, 10-methylenetetrahydrofolate; TS, thymidylate synthetase.

ities of thymidine kinase in the control group of Exp. 1 and Exp. 2 were rather different. The reason why the doses of 5-FU and its metabolites and thymidine kinase activity have various values is that the 2 experiments were undertaken independently in occasion and in separate cell lines, which had different biological characteristics. However, this discrepancy is not in conflict with our hypothesis.

In Exp. 2, the OK-432-3D group showed increased 5-FU and F-RNA concentrations and decreased levels of thymidine kinase activity, as compared to the control group. The present research also demonstrated an increased inhibition rate of TS (TSIR), no change of total TS (TStot), and a decreased tendency of free TS (TSf) in the OK-432-3D group, as compared to the control group. The elevation of TS in tumor tissue has been considered to be an important mechanism by which malignant cells may become resistant to the antitumor effects of 5-FU (27, 28). A previous report has demonstrated that exposure to 5-FU induces an acute overexpression of the target enzyme TS in the relatively resistant human colon carcinoma C1 cell line (29). Several preclinical models, both *in vitro* and *in vivo*, have confirmed a rapid increase in TS levels after flupropyrimidine therapy (30). An increase of TS has also been reported in the tumors of breast cancer patients treated with 5-FU (31). It has been also reported that, in the human colon carcinoma H630 cell line, treatment with IFN-γ obviate the 5-FU-induced TS overexpression, with a resultant reversal of 5-FU resistance (29); these results suggest that the induction of TS may represent a relevant mechanism of resistance to 5-FU. Moreover, this mechanism can be circumvented in the presence of IFN-γ. It is therefore possible that the present result, *i.e.*, that no change was induced in the TStot after intratumoral injection of OK-432, might be attributable to OK-432-induced IFN-γ production.

In contrast, another report demonstrated that the administration of IFN-α/β induced TS overexpression by the administration of 5-FU in Renca tumor, despite significant inhibition of tumor growth (32). As described above, acute induction of TS has been demonstrated as an important mechanism in the emergence of 5-FU resistance (27, 28). Therefore, an inconsistency among tumors exists as regards the relation of TS induction and enhancement of cytotoxicity. A recent report described *in vitro* synergistic cytotoxicity using IFN-γ and 5-FU, or its metabolite 5-FUdR, in the murine colon adenocar-

cinoma 38 cell line and in the HL-60 human promyelocytic leukemic cell line (33). It has also been found that IFN-γ is more potent than IFN-α/β, with regard to enhancing fluoropyrimidine cytotoxicity in the MCA-38 cell line (33). Therefore, in treating colon-26 tumor-bearing mice, IFN-γ or OK-432 may be recommended rather than IFN-α/β for the inhibition of TS induced by 5-FU. In the last few decades, clinical investigations combining 5-FU with IFN were initially undertaken using IFN-α (34, 35). In studies to investigate the mechanistic interaction between IFN-α and 5-FU, it was observed that the former resulted in an increase in the anabolism of 5-FU to its active FdUMP form; this was possibly achieved by effects on certain enzymes such as thymidine phosphorylase (36). When FdUMP binds to TS, cytotoxic effects are enhanced, and the critical locus of biochemical modulation of 5-FU by IFN-a is in the potentiation of DNA-directed toxicity through the increase of FdUMP. However, the present study demonstrated that intratumoral injection of OK-432 did not elevate the level of FdUMP. On the other hand, injection of OK-432 did increase the concentration of F-RNA in tumor tissue. These results suggest that biochemical modulation of 5-FU by OK-432 is mainly located in RNA-directed toxicity, rather than in DNA damage.

In the present study, the thymidine kinase (TK) activity in tumor tissue was also determined. TK activity in mice treated with 5-FU was significantly decreased by intratumoral injection of OK-432. TK has been recognized as being a salvage enzyme in terms of pyrimidine nucleotide biosynthesis and DNA replication (37). Previous reports have already demonstrated that the administration of IFN-α/β significantly reduced the TK activity of tumor tissue, as compared with the activity measured in untreated mice and mice treated with 5-FU (32). IFNs have been known to inhibit the incorporation of exogenous thymidine into DNA in a variety of benign and malignant cells (38). This phenomenon has previously been considered to be due to a decreased rate of DNA synthesis, which is caused by the cytostatic activity of IFNs. It has been also shown that the inhibition of proliferation of the Daudi line of human lymphoblastoid cells by human IFNs is accompanied by decreased rates of membrane transport and phosphorylation of thymidine by TK (39). It is, therefore, speculated that OK-432-induced suppression of TK activity is due to IFN-γ endogenously induced by OK-432. As described above, the administration of 5-FU induced a complex of TS and FdUMP, resulting

in the reduction of intracellular sources to the deoxythymidine monophosphate (dTMP) pool for DNA synthesis. Such conditions make tumor cells partly dependent on exogenous thymidine for their growth. The reduction of TK by administration of OK-432 or IFNs can change the utilization of exogenous thymidine, and thus may represent the mechanism of additive antitumor effects of the 2 agents, which occur via the suppression of the salvage pathway of dTMP induction.

Our results suggest that combined therapy of OK-432 followed by 5-FU administration may present a promising antitumor strategy against adenocarcinoma. This result is in agreement with a report that synergy between IFNs and 5-FU is usually observed when IFNs were administered before 5-FU rather than the reverse, suggesting that modulation of 5-FU by IFNs may be sequence dependent (40). 5-FU remains as the primary agent used in the treatment of advanced carcinoma; the response rate of this drug is 10% to 15% (41), and the tumor response to 5-FU therapy generally occurs quickly, with a median time to response of 6 weeks (42). Unfortunately, duration of response is typically short, with a median survival time of 10 to 13 months (43). After disease progression despite initial 5-FU treatment, subsequent attempts to treat patients with 5-FU-based regimens by changing the 5-FU dose, schedule, or biochemical modulator are warranted, as enhancement of the efficacy of 5-FU is anticipated. Several trials have already considered agents that biochemically modulate 5-FU to increase its efficacy. These biochemical modulators, as described above, have included leucovorin (3), IFN-α (4, 5, 35), folinic acid (44), and N-phosphonacetyl-L-aspartic acid (PALA) (45). The mechanisms by which these agents modulate 5-FU antineoplastic activity have not been fully elucidated. However, potential interactions may include increased formation of active metabolites of 5-FU by these agents or enhanced inhibition of critical enzymatic pathways. These agents seem to work independently, act in different sites and via different mechanisms. Because of the disappointing results obtained with single modulations, multimodal biochemical modulation of 5-FU would be feasible and effective in cases of advanced cancer. As shown in this study, the biochemical modulation of 5-FU by OK-432 increased the levels of F-RNA, resulting in RNA damage. The effects of this agent differs from that of other agents. Therefore, OK-432 is a promising candidate as a biochemical modulator of 5-FU. Although results of basic and clinical studies are encouraging,

prospective clinical trials are needed to establish a therapeutic regimen of multimodal biochemical modulation of 5-FU by several agents, including OK-432.

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