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Effects of Antitumor Agents on Subcutaneous Implants and Hepatic Metastases of Colon Carcinoma 26 in Mice

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We investigated the responses of experimentally produced hepatic metastases of colon carcinoma 26 tumor and subcutaneously (SC) implanted colon carcinoma 26 tumor in mice to 17 clinically used and one under-development antitumor agents using same dose regimen. In intravenous administrations on days 7 and 14, there were no significant differences in their responses to most of the tested agents. However, there were big differences in the responses to some of the agents. Nimustine more effectively prolonged the lifespan of SC implanted tumor-bearing mice than of mice bearing hepatic metastases. Mitomycin C was, however, considerably more effective on hepatic metastases than on SC implanted tumor. ME2303, a new fluorinated anthracycline derivative, showed a similar effect to doxorubicin on both tumors. However, administrations of ME2303 on days 7, 11 and 15 showed more marked antitumor effect only on hepatic metastases than administrations on days 7 and 14. Doxorubicin was less active against both tumors for administrations on days 7, 11 and 15 than for those on days 7 and 14. These results suggest the importance of the site of tumor growth for the action of some drugs. ME2303 may be active against hepatic metastases if it is administered by multiple injections.

Key words: Hepatic metastasis — Colon carcinoma 26 — Antitumor effect — Anthracycline — ME2303

As is generally known, many drugs are concentrated in the liver after administration. For example, doxorubicin level in the liver is about 100 times that in the plasma.[1] Moreover, almost all drugs are metabolized in the liver, and consequently some are inactivated, whereas others are activated. Tumors in the liver may affect liver functions. Further, tumor sensitivity to drugs may be under the control of growth site. Therefore, it is of interest to known whether particular antitumor agents are effective against tumors in the liver. To develop effective agents against tumors in the liver, several experimental models of hepatic metastases have been reported.[2–5] In this study, artificial hepatic metastases (hepatic neoplastic nodules) were produced in CDF_1 mice by intrasplenic injection of colon carcinoma 26. Micronodules of the tumor were found as early as on day 4 postimplantation in our previous study.[6] Furthermore, to examine the difference in antitumor activity of 17 clinically used agents and a new fluorinated anthracycline derivative under development (ME2303, (8*S*,10*S*)-8-(6-carboxyhexanoyloxyacetyl)-10-[(2,6-dideoxy-2-fluoro-α-L-talopyranosyl)oxy-7,8,9,10-tetrahydro-6,8,11-trihydroxyl-1-methoxy-5,12-naphthacenedione) on tumors at different sites, the antitumor effects (ILS%) on hepatic metastases of colon carcinoma 26 were compared with those on SC implanted tumor. ME2303 is known to be effective against artificial hepatic metastases of colon carcinoma 26.[6]

MATERIALS AND METHODS

Chemicals We used actinomycin D (Banyu Pharm. Co., Ltd., Tokyo), bleomycin (Nippon Kayaku Co., Ltd., Tokyo), cisplatin (Bristol-Myers Squibb K.K., Tokyo), cyclophosphamide, vincristine and vindesine (Shionogi & Co., Ltd., Osaka), daunorubicin, pirarubicin and ME2303 (Meiji Seika Kaisha, Ltd., Tokyo), doxorubicin, 5-fluorouracil, mitomycin C and epirubicin (Kyowa Hakko Kogyo Co., Ltd., Tokyo), doxifluridine (Nippon Roche, Kamakura), 5-fluoro-2′-deoxyuridine (Sigma Chemical Co., St. Louis, MO), methotrexate and mitoxantrone (Lederle Japan, Tokyo) and nimustine (Sankyo & Co., Ltd., Tokyo).

Animals Groups of six or more specific-pathogen-free male CDF_1 (BALB/c×DBA/2) mice weighing 22 to 24 g (Japan SLC, Inc., Hamamatsu) were housed in plastic cages with woodchip bedding and were fed CA-1 pellet diets (CLEA Japan, Inc., Tokyo). Feed and water were available *ad libitum*. All experiments were performed in an animal laboratory with controlled temperature (25°C).

The antitumor effects of the drugs on subcutaneous (SC) tumors were evaluated for 6 CDF_1 males per group. Cells (5×10^5) of colon carcinoma 26 were implanted SC on day 0 in the right thigh.

Experimental procedure for inducing artificial liver metastases Colon carcinoma 26 was maintained in male BALB/c mice. Suspensions of colon carcinoma 26 cells

in saline were prepared from surgically removed tumors by disaggregating the tumor pieces by gentle homogenization in a glass homogenizer and sieving the cell suspension through a 120 mesh sieve. Artificial multiple hepatic metastases were produced according to the method of Kopper *et al.*[7] Briefly, mice were anesthetized with ether and a left subcostal incision (about 5 mm) was made to externalize the spleen. A 27-gauge needle (Terumo Japan, Tokyo) was used to puncture the splenic capsule and 5×10^4 viable tumor cells in 0.1 ml of saline solution were injected directly into the upper pole of the spleen. The number and viability were determined by trypan blue exclusion. Gentle pressure was applied for a period of 10 s to prevent hemorrhage and tumor cell leakage. The artery and vein lienalis were then clamped with a medium hemoclip (Edward Weck & Co., Inc., NC) and the spleen was removed. The abdomen was surgically sutured and the skin was closed with disposable skin clip applicators (Avlox 12, Medi Plast, Sweden). The mice were allowed to recover (4 days) and randomized before being distributed in cages (six or more mice/group).

Drug treatment Mice inoculated SC or by intrasplenic injection were intravenously administered the drugs at their maximum tolerated doses on days 7 and 14 or on days 7, 11 and 15. Maximum tolerated dose was determined in SC tumor-bearing mice. The injection volume was 0.01 ml/g body weight. Observation was terminated on day 100. All mice surviving to day 100 were recorded as "cured." The antitumor effect (ILS%) was evaluated by comparing the mean survival time of animals in each group with that of control group. Dead artificial hepatic metastasis-bearing mice were examined for the presence of metastases.

Biochemical tests of the plasma Plasma samples without signs of hemolysis were stored at −20°C until analysis. Alkaline phosphatase (AP), lactic dehydrogenase (LDH), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), leucine aminopeptidase (LAP), and γ-glutamyl transpeptidase (γGTP) were biochemically tested using *p*-nitrophenylphosphate (Bessey-Lowry method), pyruvic acid (UV), L-aspartate (UV), L-alanine (UV), L-leucyl-*p*-nitroanilide and γ-glutamyl-*p*-nitroanilide, respectively. These enzymes were chosen because alterations of these enzymes can reliably indicate the presence of hepatic metastases in humans.[8]

RESULTS

Biochemical tests of plasma from mice bearing SC implanted tumor or hepatic metastases of colon carcinoma 26 Histologically well-circumscribed tumors had been found on day 8 following intrasplenic injection of colon carcinoma 26.[6] At this time, the plasma GOT, GPT and LDH levels in the hepatic metastasis-bearing mice were significantly abnormal (high) compared to SC tumor-bearing mice and normal mice (Fig. 1). The mean GOT levels were 38±6 (SD) (n=8), 63±3 (n=3) (1.6-fold compared to normal mice) and 661±421 IU/liter (n=4) (17.2-fold) respectively in normal, SC tumor-bearing and hepatic metastasis-bearing mice. The mean GPT levels were 25±5, 16±1 (0.6-fold) and 396±230 IU/liter (15.7-fold) in normal, SC tumor-bearing and hepatic metastasis-bearing mice, respectively. The mean LDH levels were 271±49, 2003±114 (7.4-fold) and 5740±2031 IU/liter (21.2-fold) in normal, SC tumor-bearing and hepatic metastasis-bearing mice, respectively. However, AP, LAP and γGTP levels were not different between normal and tumor-bearing mice.

Effects of various antitumor agents on hepatic metastases of colon carcinoma 26-bearing mice The range of lifespan of untreated CDF_1 mice bearing hepatic metastases was narrow; in 11 experiments, the mean survival time of the control group ranged from 14 to 21 days (range: 13 to 25 days). All the dead animals had many metastases only in the liver (liver weight of untreated control: 5.77±1.22 g, n=77). The effects of 17 antitumor agents against these established multiple murine hepatic metastases of colon carcinoma 26 are shown in Table I. Nimustine, a nitrosourea derivative, showed the best ILS of 271% at the dose of 25 mg/kg, followed by cyclophosphamide (146%) at 100 mg/kg, and doxorubicin (138%) at 12.5 mg/kg. Among the 4 anthracycline derivatives, doxorubicin showed the best ILS%.

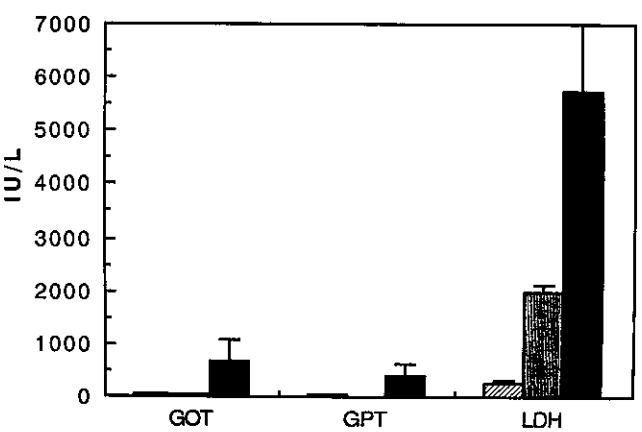

Fig. 1. Plasma levels of GOT, GPT and LDH in normal, SC tumor- and liver metastasis-bearing mice (mean±SD, n=8, 3 and 4 mice, respectively). ▨, Normal CDF_1; ▩, 8-day-old SC implanted Co 26 carcinoma-bearing mice; ■, 8-day-old artificial hepatic metastasis-bearing mice.

Table I. Effects of Antitumor Agents on Hepatic Metastases of Colon Carcinoma 26

	Antitumor agent	Dose (mg/kg)	MST (days)	ILS (%)
I	Control		21.1±1.4[a]	—
	Daunorubicin	12.5	31.2±2.5**	48
	Doxorubicin	12.5	50.2±6.1***	138
	Epirubicin	12.5	31.0±2.9**	47
	Pirarubicin	12.5	32.7±3.0**	55
	Mitomycin C	5	35.7±2.5***	69
	Cisplatin	5	34.8±3.2**	65
II	Control		15.7±0.8	—
	Actinomycin D	0.5	20.3±0.9**	30
	Mitoxantrone	2.5	18.7±1.6	19
	5-Fluorodeoxy-uridine	500	24.2±1.1***	30
	Methotrexate	50	19.8±2.4	22
	Nimustine	12.5	47.0±3.6***	199
		25	58.2±7.3*** (1/6)[b]	271
	Cyclophos-phamide	100	38.7±1.8***	146
III	Control		18.5±0.7	—
	Bleomycin	100	27.5±3.1*	49
	Vincristine	1	20.8±1.8	12
	Vindesine	2.5	26.2±3.1*	42
	5-Fluorouracil	200	23.9±0.5***	29
	Doxifluridine	500	23.8±2.8	29

a) Mean±SE of dead mice.
b) Number of cured mice per treated mice, on day 100.
*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ compared to control.

Table II. Effects of Antitumor Agents on Subcutaneously Implanted Colon Carcinoma 26

	Antitumor agent	Dose (mg/kg)	MST (days)	ILS (%)
I	Control		25.5±2.3[a]	—
	Daunorubicin	12.5	43.8±1.9***	72
	Doxorubicin	12.5	43.0±3.0**	69
	Epirubicin	12.5	40.5±2.7**	59
	Pirarubicin	12.5	51.3±5.3***	101
	Bleomycin	100	33.3±1.3*	31
	Mitomycin C	5	32.0±4.8	25
	Vincristine	1	30.3±1.6	19
	5-Fluorouracil	200	34.3±2.0*	35
	5-Fluorodeoxy-uridine	500	36.8±2.6**	44
	Methotrexate	50	32.4±1.0*	27
	Cisplatin	5	36.8±2.8**	44
	Cyclophos-phamide	50	38.7±2.3**	52
	Nimustine	12.5	58.5±7.9** (2/6)[b]	129
II	Control		27.4±2.0	—
	Actinomycin D	0.5	35.0±2.6*	28
	Vindesine	2.5	36.3±3.3*	32
	Mitoxantrone	2.5	33.8±4.5	23
	Doxifluridine	500	28.7±1.9	5
	Cyclophosphamide	100	44.0±2.5***	61
	Nimustine	25	>100 (5/6)[b]	

a) Mean±SE of dead mice.
b) Number of cured mice per treated mice, on day 100.
*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ compared to control.

Effects of various antitumor agents on SC implanted colon carcinoma 26-bearing mice The mean survival time in the control group was approximately 26 days (range: 19 to 35 days). The mean ILS% values of the 17 antitumor agents are as summarized in Table II. Nimustine was more active against SC-implanted tumor than against tumors in the liver. SC tumor completely regressed in 2 of 6 animals even at 12.5 mg/kg of nimustine. At the dose of 25 mg/kg, there were 5 cured mice out of 6 SC tumor-bearing mice while one of 6 animals bearing tumors in the liver was cured. All the anthracycline derivatives and cyclophosphamide were active (more than 50% ILS) against this tumor. The other drugs showed ILS of less than 40%.

The effects of antitumor agents were compared on SC tumor and the hepatic metastases to find active agents against the tumor in the liver. Table III summarizes the activity of the drugs tested. In addition to the data of Tables I and II, Table III includes the results of another experiment. Moreover, the ratios of ILS% for the hepatic metastases to that for the SC tumor-bearing mice were calculated. These ratios ranged from about 0.7 to 2.0. The maximum ratio was 2.7 for mitomycin C. In terms of relative therapeutic efficacy against hepatic metastases compared with that against SC-implanted tumor, this agent was superior to the other agents. (Mitomycin-C produced significant effect on hepatic metastases, but not on SC tumor, as shown in Tables I and II.)

Effect of ME2303 on hepatic metastases and SC tumor Administrations of ME2303 on days 7 and 14 revealed almost the same activity against hepatic metastases and SC tumor as seen for doxorubicin (Table IV and V). In administrations on days 7, 11 and 15 at 50 mg/kg/day, ME2303 showed better ILS% than in administrations on days 7 and 14 at 100 mg/kg/day and some mice were cured (Table IV). However, ME2303 showed inferior ILS% against SC tumor when administered on days 7, 11 and 15 than when administered on days 7 and 14 (Table

V). On the other hand, the antitumor effect of doxorubicin was weaker against both tumors in administrations on days 7, 11 and 15 than on days 7 and 14. Table VI summarizes the comparative response of hepatic metastases and SC-implanted colon carcinoma 26 to doxorubicin and ME2303. Table VI includes the data of Tables IV and V and several more experiments. The maximum ratio of ILS% for the hepatic metastases to that of the SC tumor-bearing mice was given by the 3-fraction dose regimen of ME2303 and its value was 9.1.

Table III. Increase of Life-span by Various Antitumor Agents in Hepatic Metastases and SC Tumor-bearing Mice

Antitumor agent	Dose (mg/kg)	Mean ILS (%) Hepatic metastases (A)	Mean ILS (%) SC tumor (B)	A/B ratio
Daunorubicin	12.5	70±22[a]	54±19	1.3
Doxorubicin	12.5	111±17	55±9	2.0
Epirubicin	12.5	56±9	59	1.0
Pirarubicin	12.5	83±16	70±31	1.2
Bleomycin	100	49	31	1.5
Actinomycin D	0.5	30	28	1.1
Mitomycin C	5	52±11	19±7	2.7
Vincristine	1	18±6	25±6	0.7
Vindesine	2.5	49±7	23±9	2.1
Mitoxantrone	2.5	19	23	0.8
5-Fluoro-uracil	200	29	25±10	1.2
5-Fluorodeoxy-uridine	500	30	39±5	0.8
Doxifluridine	500	29	5	—
Methotrexate	50	22	27	0.8
Cisplatin	5	53±10	32±6	1.5
Cyclophos-phamide	50	111	52	2.1
	100	146	61	2.4
Nimustine	12.5	199	129 (2/6)[b]	<1.5
	25	269 (1/6)[b]	(5/6)[b]	—

The antitumor agents were administered at the maximum tolerated doses.
a) ILS (%) of dead mice. Mean±SE of 2 or 3 experiments.
b) Number of cured mice per tested mice. The number of cured mice was determined at day 100 of the observation period.

Table IV. Effects of Doxorubicin and ME2303 on Hepatic Metastases of Colon Carcinoma 26

Antitumor agent	Dose (mg/kg)	Treatment schedule	MST (days)	ILS (%)
Control			19.7±0.9[a]	
Doxorubicin	12.5	Days 7, 14	42.6±2.2***	116
	6.3	Days 7, 11, 15	30.4±2.9*	54
ME2303	100	Days 7, 14	44.3±3.9***	125
	50	Days 7, 11, 15	61.0±9.7*** (1/6)[b]	210

a) Mean±SE of dead mice.
b) Number of cured mice per treated mice, on day 100.
*, $P<0.05$; ***, $P<0.001$ compared to control.

Table V. Effects of Doxorubicin and ME2303 on Subcutaneously Implanted Colon Carcinoma 26

Antitumor agent	Dose (mg/kg)	Treatment schedule	MST (days)	ILS (%)
Control			29.2±2.7[a]	
Doxorubicin	12.5	Days 7, 14	40.3±3.0**	38
	6.3	Days 7, 11, 15	33.1±3.8	13
ME2303	100	Days 7, 14	39.4±4.1*	35
	50	Days 7, 11, 15	36.3±1.9	24

a) Mean±SE of six mice.
*, $P<0.05$; **, $P<0.01$ compared to control.

Table VI. Comparison of Antitumor Activities of Doxorubicin and ME2303

Antitumor agent	Dose (mg/kg/day)	Schedule	Mean ILS (%) Hepatic metastases (A)	Mean ILS (%) SC tumor (B)	A/B ratio
Doxorubicin	12.5	Days 7, 14	111±17[a]	55±9	2.0
	6.3	Days 7, 11, 15	44±10	14±1	3.1
ME2303	100	Days 7, 14	118±8	53±16	2.2
	50	Days 7, 11, 15	201±10	22±2	9.1

a) ILS (%) of dead mice. Mean±SE of 2 or 3 experiments.

This regimen was significantly active against hepatic metastases (Table IV), but not SC tumor (Table V). Thus, the 3-fraction dose regimen of ME2303 at 50 mg/kg/day showed the best efficacy against hepatic metastases.

DISCUSSION

Hepatic nodules of colon carcinoma 26 were produced by splenic injection of the tumor. During the autopsy of dead animals, no visible tumor growth could be detected in organs other than the liver. The hepatic nodules resulting from splenic injection of tumor cells are considered as artificial metastases. In this investigation, we used this system to evaluate the response of the hepatic metastases to chemotherapy. Moreover, it was considered that to compare the effects of the drugs on SC tumor and on the hepatic metastases might provide novel information that would be helpful in therapy for hepatic metastases, and might help us to select potentially active antitumor agents for liver tumor. In hepatic metastasis-bearing mice high plasma GOT, GPT and LDH levels were observed. This suggests that the presence of a tumor in the liver may affect hepatic functions. There were no large differences in the antitumor effects of most agents on SC tumor and hepatic metastases, though some drugs did show marked differences. The maximum ILS% ratio in the mice bearing hepatic metastases to the SC tumor-bearing mice was 2.7 for mitomycin C administered on days 7 and 14. Namely, with the same administration schedule, this drug markedly prolonged the survival period of mice bearing hepatic metastases compared to SC tumor-bearing mice. Moreover, the 3-fraction dose regimen (days 7, 11 and 15) of ME2303 produced greater efficacy against the hepatic metastases but not against SC tumor, than the 2-fraction dose regimen (days 7 and 14) ($P<0.01$). The ratio of ILS% for the hepatic metastases to that for the SC tumor-bearing mice was 9.1. This value is greater than that of mitomycin C. However, the effectiveness of doxorubicin did not rise in the 3-fraction dose regimen. These results show that administration schedule markedly affects activity against the tumor in the liver.

5-Fluorouracil and mitomycin C have been used clinically in the intraarterial treatment of liver tumors.[9] Our study suggests that fractionated administration of ME2303 may be a superior therapy for liver tumors. Furthermore, comparison of the effects of an antitumor agent on SC-implanted tumor and the same tumor in the liver may be advantageous for *in vivo* screening of newly developed agents effective against tumors in the liver.

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