

## Prevention of Hepatic and Peritoneal Metastases by the Angiogenesis Inhibitor FR-118487 after Removal of Growing Tumor in Mice

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We established a mouse “primary tumor resection model” in which a transplanted tumor was resected after an orthotopic transplantation of colorectal cancer tissue to estimate the therapeutic effect of an angiogenesis inhibitor on metastasis. The angiogenesis inhibitor FR-118487 is a member of the fumagillin family. Here, 1 mg/kg/day of FR-118487 was subcutaneously administered to nude mice for 1 week, 2 weeks, or 4 weeks through an osmotic pump. Liver metastasis developed in 7 of 9 control mice, 2 of 6 mice that underwent the tumor resection 2 weeks after transplantation (early resection), and in all 7 of the mice that underwent the tumor resection 4 weeks after transplantation (late resection). In the short treatment trial, the FR-118487 administration immediately after the early resection completely inhibited both hepatic and peritoneal metastases, whereas its administration after the late resection had no effect on liver metastasis. In the prolonged treatment trial, inhibitory effects of prolonged treatment with FR-118487 on both hepatic and peritoneal metastases after the late resection were clearly demonstrated. The mice of the resection-alone group all died within 106 days after tumor inoculation, due to metastases of colon carcinoma. In contrast, half of the mice that underwent resection and then received antiangiogenic therapy were alive at the end of the observation period (160 days after transplantation). In conclusion, the combination of surgery and subsequent antiangiogenic therapy may be useful to prevent the distant metastasis of colorectal cancer and to improve the prognosis of patients with colorectal cancer.

Key words: Hepatic metastasis — Metastasis after surgery — Human colon cancer — Angiogenesis inhibitor — Tumor removal

Hepatic metastasis is the most common cause of death after surgery for patients with colorectal cancer, and methodologies to prevent liver metastasis after surgery have been sought to improve the prognosis of these patients. There are no reports of a liver metastasis model after the resection of a primary colorectal tumor, to our knowledge. We established such a metastatic model, which well reflected the clinical situation, in which hepatic metastases developed after the removal of transplanted orthotopically-growing colorectal tumor. Accordingly, the results obtained in this model are thought to be of relevance to the clinical situation. With this model, therapeutic effects of antiangiogenic therapy followed by surgery on human colon cancer have been investigated.

Tumor neovascularization is part of the complicated process of tumor metastasis,<sup>1</sup> and is crucial to the occurrence of hepatic metastasis.<sup>2</sup> Since a small focus of tumor cells cannot grow indefinitely at a secondary site without the induction of angiogenesis, it is speculated that the inhibition of angiogenesis could provide a potent form of therapy for the hepatic metastasis of colonic cancer.<sup>3</sup> Many studies have demonstrated the essential role of angiogenesis

in the aggressive growth of solid tumors and their metastases.<sup>1,4–6</sup> We showed previously that a s.c. injection of the angiogenesis inhibitor TNP-470 prevented hepatic metastases of human gastrointestinal carcinomas in nude mice.<sup>7–9</sup>

In the present study, the therapeutic effect of the angiogenesis inhibitor FR-118487 was investigated by using this metastatic model. FR-118487 was obtained by the chemical modification of FR-111142, which was isolated from the fermentation products of *Scolecobasidium arenarium* F-2015. FR-118487 has about 5–10 times stronger antiangiogenic activities than the parent compound and inhibited the angiogenesis in a rabbit cornea assay and suppressed solid tumor growth in mice. The chemical structure and antiangiogenic activities of FR-118487 resemble those of TNP-470,<sup>10,11</sup> and FR-118487 is thus classified as a member of the fumagillin family.

### MATERIALS AND METHODS

**Materials** FR-118487 was a kind gift from Fujisawa Pharmaceutical Co., Ltd. (Tokyo). Its structure has already been reported.<sup>10</sup> FR-118487 was dissolved in propylene glycol. The ALZET osmotic pump was purchased from ALZA Co. (Palo Alto, CA). FR-118487 is regarded

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as being a member of the fumagillin (Fig. 1), based on physico-chemical data.<sup>10)</sup>

**Human colon cancer xenografts** The human colon cancer xenograft TK-4 was used in the present study. TK-4 was established in our department in 1993 and maintained by means of s.c. passage.<sup>12)</sup>

**Animals** Male BALB/c-*nu/nu* mice were obtained from Clea Japan, Inc. (Tokyo). Animals were used at 6 weeks of age.

**Orthotopic transplantation of colon cancer xenografts, and resection** The method of orthotopic transplantation has been reported elsewhere.<sup>7)</sup> Briefly, small pieces of TK-4 tumor tissue were resected aseptically during the exponential growth phase from tumor subcutaneously implanted in nude mice. The cecum was carefully exteriorized, and the serosa was injured at the site where the tumor was to be implanted. A tumor piece was then fixed on each injured site of the serosal surface with a 6-0 Vicryl transmural suture (Ethicon, Inc., Somerville, NJ). The intestine was returned to the abdominal cavity, and the abdominal wall and skin were closed with 6-0 Vicryl sutures. Two weeks (early resection) or 4 weeks (late resection) after the orthotopic transplantation, the cecum with the growing tumor was exteriorized and removed using a Surgiclip (Auto Suture Japan Co., Tokyo).

**Inhibitory effect of FR-118487 on tumor growth and hepatic metastasis without surgery** To evaluate the therapeutic effect of FR-118487 on the tumor growth and liver metastasis, animals were given FR-118487 s.c. at a dose of 1 mg/kg/day for 14 days starting on the 10th day after the orthotopic transplantation, via an osmotic pump. As a control, the same volume of physiological saline was given to other mice.

All of the mice were sacrificed 6 weeks after the transplantation. An autopsy was performed immediately, and the tumors growing on the cecal wall were removed and weighed. The liver was processed for routine histological examination to detect metastases after careful macroscopic examination.

**Therapeutic effect of FR-118487 on peritoneal and hepatic metastases after removal of the primary tumor**

Animals were randomized into five groups. Early resection-alone group (ER group), late resection-alone group (LR group), early resection followed by FR-118487 treatment (early FR group), and late resection followed by FR-118487 treatment (late FR group). FR-118487 was administered to the mice s.c. at a dose of 1 mg/kg/day for 7 days starting on the third day after surgery, by the ALZET osmotic pump implanted subcutaneously. The pumps were removed 8 days after their s.c. implantation. The mice of the control group were sacrificed 6 weeks after tumor inoculation. Treated mice were sacrificed 4 weeks after removal of the tumor. Autopsies were performed immediately, and the tumors growing on the peritoneal wall and

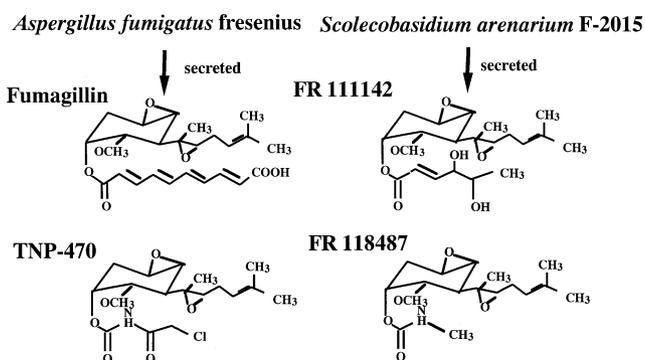


Fig. 1. Illustration of chemical structures of FR-118487 and TNP-470.

the livers were removed and processed for routine histological examination to detect metastases after careful macroscopic examination.

**Anti-metastatic effects of prolonged treatment with an angiogenesis inhibitor after removal of the primary tumor**

We estimated that prolonged treatment with an angiogenesis inhibitor after late resection of the primary tumor was effective for prevention of metastases. The cecum with the growing tumor was removed 4 weeks after the orthotopic transplantation. Animals in the treated group were given FR-118487 s.c. at a dose of 1 mg/kg/day for 28 days starting on the third day after surgery, with an ALZET osmotic pump implanted subcutaneously. Mice were sacrificed 30 days after removal of the tumor.

**Effect of angiogenesis inhibitor following surgery on survival** Animals were randomized into two groups; a control group which underwent surgical removal of the cecum with the growing tumor 3 weeks after tumor implantation, and the FR group which underwent surgery 3 weeks after transplantation and received antiangiogenic therapy for 7 days starting on the third day after surgery, via an osmotic pump. This experiment continued for over 5 months.

**Statistical analysis** Survival curves were calculated according to the Kaplan-Meier method. Student's *t* test and the  $\chi^2$  test were used for the statistical analysis.

Table I. Inhibitory Effect of the Angiogenesis Inhibitor FR-118487 on Tumor Growth and Hepatic Metastasis in Nude Mice

Group	Tumor weight (g)	Hepatic metastases No. of mice (%)	No. of metastatic foci
Control	0.56±0.18	6/8 (75.0)	6±2.5
Treated	0.35±0.06 <sup>a)</sup>	1/8 (12.5) <sup>b)</sup>	2

The data are mean±SD of eight mice per group.

a) *P*<0.001 versus control group.

b) *P*<0.05 versus control group.

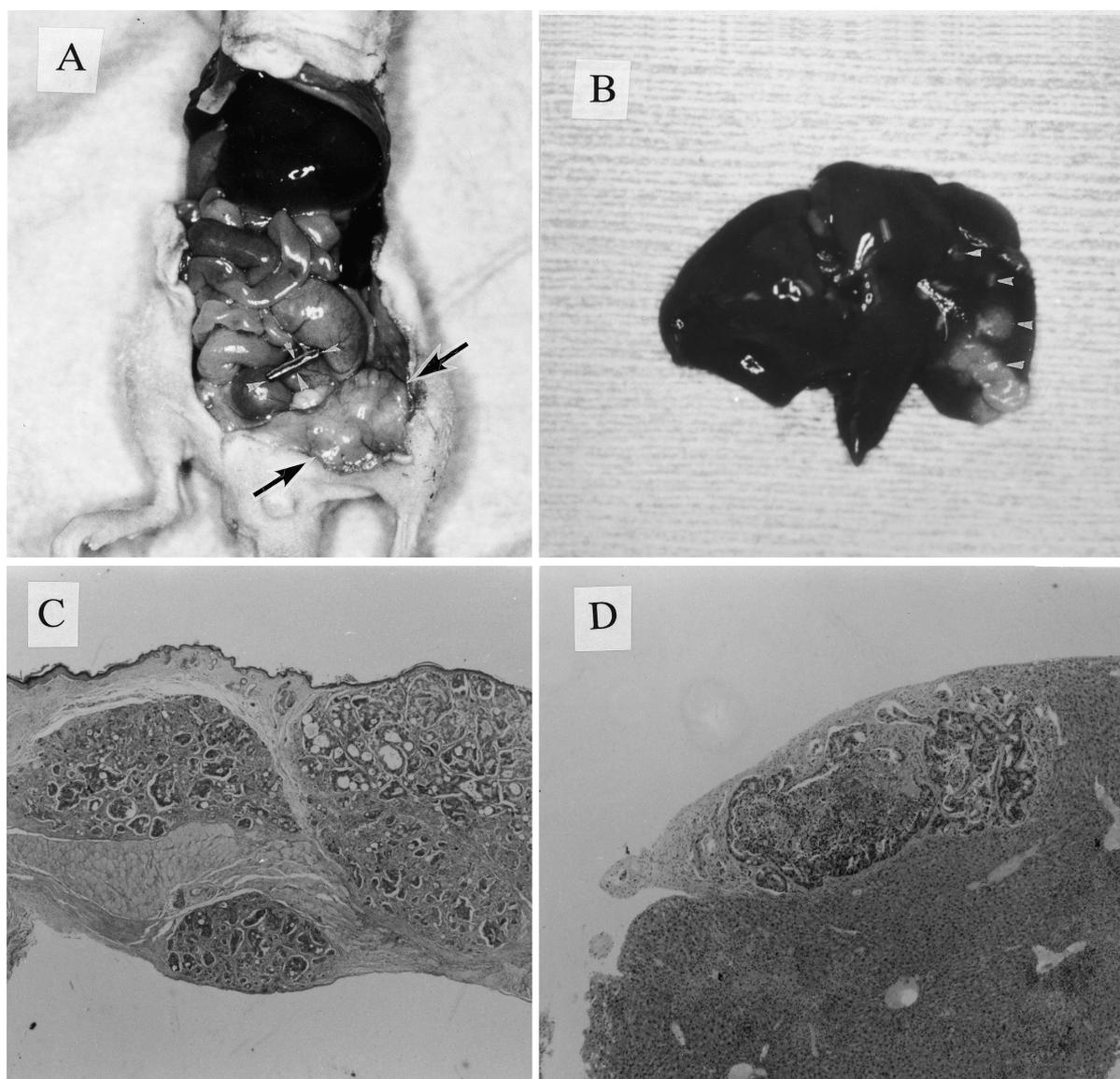


Fig. 2. (A) Abdominal cavity of a nude mouse from the resection-alone group. The white arrow indicates the surgical clip at the cut end of the cecum. No intraperitoneal adhesion is observed. The black arrow indicates peritoneal metastases. (B) Liver of a nude mouse that had undergone the removal of a cecal tumor. The arrow indicates foci of hepatic metastases. (C) A photomicrograph of the TK-4 tumor growing in the peritoneum that is shown in (A). (D) A photomicrograph of the metastatic focus in the liver that is shown in (B). The tissue was fixed, embedded, sectioned, and stained with H&E using standard procedures.

## RESULTS

### Therapeutic effect of FR-118487 on tumor growth and hepatic metastasis without surgery

A therapeutic effect of FR-118487 on both tumor growth and hepatic metastasis was clearly demonstrated compared with the control group (Table I). Six mice (6/8, 75.0%) with hepatic metastasis were observed in the control group, whereas only one mouse (1/8, 12.5%) showed hepatic metastasis in

the treated group. The FR-118487 administration thus significantly decreased the number of metastatic foci in the liver (Table I).

### Therapeutic effect of FR-118487 on the hepatic and peritoneal metastases after removal of primary tumor

Both the hepatic and peritoneal metastases in the "primary tumor removal" mice could be identified macroscopically. The macroscopic appearance and histological findings are demonstrated in Fig. 2. Though seven mice (7/9, 77.8%)

Table II. Inhibitory Effect of FR-118487 on Hepatic and Peritoneal Metastases after the Removal of the Primary Tumor

Group	Resection <sup>a)</sup> (days)	FR-118487	Hepatic metastases No. of mice (%)	Peritoneal dissemination No. of mice (%)
Control	—	—	7/9 (77.8)	
Early resection	14	—	2/6 (33.3)	6/6 (100)
Early FR	14	+	0/6 (0) <sup>b)</sup>	0/6 (0) <sup>c)</sup>
Late resection	28	—	7/7 (100)	7/7 (100)
Late FR	28	+	6/6 (100)	1/6 (16.7) <sup>d)</sup>

a) Days after tumor implantation.

b)  $P < 0.01$  versus control, late resection and late FR groups.

c)  $P < 0.05$  versus resection-alone groups.

d)  $P < 0.05$  versus resection-alone groups.

Table III. Antimetastatic Effect of Prolonged Treatment with an Angiogenesis Inhibitor after the Removal of the Primary Tumor

Group	Liver metastases No. of mice (%)	Peritoneal dissemination No. of mice (%)
Control	9/10 (90)	8/10 (80)
Medication	2/10 (20) <sup>a)</sup>	0/10 (0) <sup>a)</sup>

a)  $P < 0.01$  versus control.

with hepatic metastasis were observed in the control group, only two mice (2/6, 33.3%) in the ER group showed hepatic metastasis. Early resection of the tumor thus had a tendency to inhibit the hepatic metastasis of colon cancer. In the six mice of the early FR group, hepatic metastasis was never observed. In contrast, hepatic metastasis developed in all six of the LR mice; FR-118487 had no inhibitory effect on hepatic metastasis in this group (Table II). Interestingly, peritoneal dissemination developed in all mice of both the ER and LR groups, and the FR-118487 administration following surgery significantly inhibited peritoneal dissemination in both groups (Table II).

**Anti-metastatic effects of prolonged treatment with an angiogenesis inhibitor after removal of the primary tumor** Because of the absence of the effect on hepatic metastasis in the late FR group, we investigated anti-metastatic effects of prolonged treatment with an angiogenesis inhibitor after surgery. A therapeutic effect of prolonged treatment on both hepatic and peritoneal metastases was clearly demonstrated compared with the control group (Table III). Nine mice (9/10, 90%) with hepatic metastasis and eight mice (8/10, 80%) with peritoneal metastasis were observed in the control group, whereas only two mice (2/10, 20%) showed hepatic metastasis in the prolonged treatment group. Peritoneal metastases after surgery were completely prevented by prolonged treatment

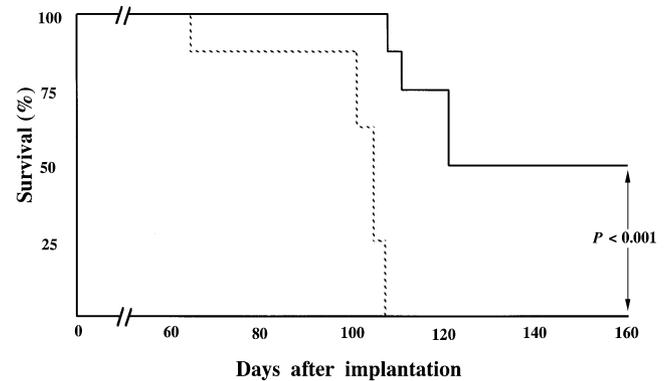


Fig. 3. Survival of mice treated with tumor removal and angiogenesis inhibitor. All eight of the mice in the resection-alone control group died within 106 days after tumor inoculation (dotted line), whereas half of the mice administered FR-118487 after surgery were alive at the end of the 160-day observation period (solid line). The survival curves were calculated according to the Kaplan-Meier method.

with an angiogenesis inhibitor. Although no inhibitory effects of short treatment (7 days) for metastases after removal of progressive tumors were observed, the efficacy of prolonged treatment (28 days) on the progressive stage of colon cancer was shown.

**Therapeutic effect of FR-118487 following surgery on survival** The mice of the resection-alone control group all died within 106 days after the tumor inoculation due to hepatic and/or peritoneal metastases of colon carcinoma. In contrast, all the mice administered FR-118487 after surgery were alive at 106 days after the tumor inoculation, and half of them were still alive at the end of the 160-day observation period (Fig. 3). The deaths in this group were due to metastases of colon carcinoma.

**Body weight** In the mice treated with 2 weeks of FR-118487, a significant decrease of body weight gain was observed compared with the control group (Fig. 4). In the mice treated with FR-118487 after surgery, no decrease in the body weight gain was observed compared with the control group at the end of the observation period (Fig. 5). In the early FR group, a significant increase ( $P < 0.01$ ) of weight gain ( $24.58 \pm 0.91$  g) was seen compared with the late FR group ( $21.27 \pm 2.17$  g). Despite the decrease in weight gain observed in the early FR group compared with the control group (data not shown), the early FR mice had gained weight satisfactorily at the completion of the administration of FR-118487.

## DISCUSSION

In our previous studies, we investigated the effect of the angiogenesis inhibitor TNP-470 on hepatic metastases of

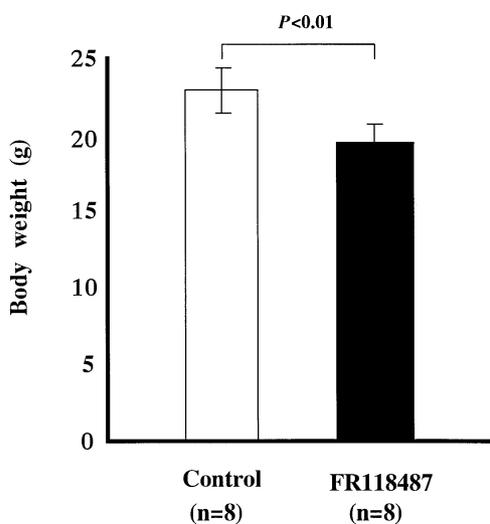


Fig. 4. Body weight of nude mice 6 weeks after tumor implantation. When FR-118487 was administered to tumor-bearing mice, it affected body weight gain. Error bars indicate the standard deviation.

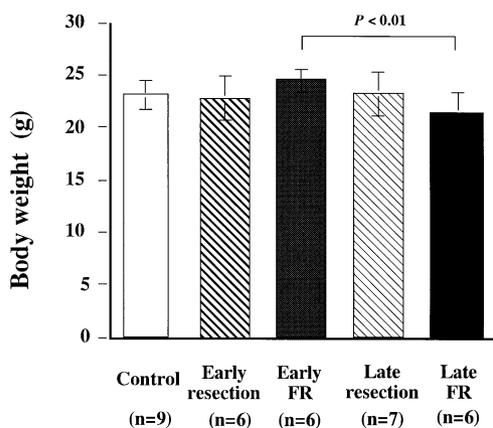


Fig. 5. Body weights of nude mice 4 weeks after tumor removal. In the mice treated with FR-118487 after surgery, a decrease in the body weight gain was not observed compared with the control group. In the early FR group, a significant increase of weight gain was observed compared with the late FR group. Error bars indicate the standard deviation.

human colorectal cancer by using the orthotopic transplantation model employed in the present study. TNP-470 did not show an inhibitory effect on the growth of tumors transplanted orthotopically.<sup>7-9</sup> In contrast, the continuous infusion of FR-118487 for 14 days inhibited the tumor growth by 62.5% compared with the control group, although both FR-118487 and TNP-470 are classified as

belonging to the fumagillin family (Fig. 1), and FR-118487 shows pharmacological action similar to that of TNP-470. Boehm *et al.* recently reported that repeated cycles of antiangiogenic therapy led to primary tumor growth shrinkage.<sup>13</sup> Sim *et al.* showed that systemic administration of recombinant angiostatin protein at doses of 1.5 mg/kg suppressed the growth of mouse lung metastases and doses of 100 mg/kg also suppressed the growth of primary tumors.<sup>14</sup> These results suggest that the continuous infusion of FR-118487 might have a more potent effect on the tumor growth compared to a bolus injection.

The antimetastatic effect of FR-118487 was clearly demonstrated in the present study. TNP-470 also has such an effect. Fumagillin derivatives seemed to have a predominantly antimetastatic effect rather than an inhibitory effect on tumor growth, as observed in experiments with TNP-470.<sup>7-9</sup> At the metastatic site, neoangiogenesis may be more crucial for the survival of tumor cells than for the primary tumor, because without angiogenesis, tumor cells cannot form “a mass” of more than a few millimeters in diameter, resulting in the induction of apoptosis or elimination by host-immunity. There is no doubt that the inhibition of the primary tumor growth is beneficial to the prevention of metastasis, because the opportunity for tumor cells to enter the circulation may be reduced by the inhibition of neoangiogenesis in the primary tumor.<sup>15, 16</sup> However, a previous study concerning the difference in the antimetastatic effects of an angiogenesis inhibitor and an antineoplastic agent (mitomycin C) showed only a marginal inhibitory effect of the antineoplastic agent on liver metastasis, although it markedly inhibited the tumor growth.<sup>8</sup> These results suggest that fumagillin derivatives should be used clinically not to shrink the tumor, but to control distant metastasis and thereby to improve the patient’s prognosis. Generally, the removal of the primary lesions is regarded as essential in the treatment of colorectal cancer. However, 10% of patients who undergo curative operation develop liver metastasis,<sup>17</sup> and 62–75% of them die within about 5 years.<sup>18</sup>

We established a primary tumor removal model that was based on the orthotopic transplantation model and mimicked the clinical situation of treatment for colorectal cancer. Kuo *et al.* reported that when primary tumors were resected 10 days after the initial orthotopic transplantation in the nude mouse cecum, neither local recurrence nor liver metastasis was observed in the treated animals.<sup>19</sup> However, no vessel was observed in the resected cecum 10 days after transplantation. They found that it was difficult to resect the cecum 14 days after transplantation, due to intraperitoneal adhesion. Although we also could not remove the cecum including the growing tumor at 14 days after orthotopic transplantation in nude mice in our initial experiments, we succeeded in cecal removal at 28 days

after transplantation because the tumor was fixed to the peritoneum of the abdominal wall by a suture. Adhesions consisting of the intraperitoneal tumor and mesenteries are very difficult to lyse. Such adhesion was prevented by fixing the implanted tumor to the peritoneum. We were able to investigate hepatic metastases of human colon cancer after removal of the primary tumor by this method. Because a tendency of inhibition of hepatic metastases was observed in the early resection group, micrometastases of liver might develop before 14 days after orthotopic transplantation. In the early FR group, which was given the angiogenesis inhibitor after early tumor removal, hepatic metastasis was completely prevented. The administration of the angiogenesis inhibitor was very effective in controlling micrometastases. In contrast, no inhibitory effect on hepatic metastasis was observed when FR-118487 was administered after a late resection in the first trial. Angiogenesis inhibitors produced by a primary tumor can create a systemic antiangiogenic environment and maintain metastatic tumor cells in a state of dormancy.<sup>4, 20</sup> Metastatic tumor cells of the liver might escape from dormancy after the removal of a large tumor on the cecum. The features of fumagillin derivatives seem to be well-demonstrated in these results, i.e., they are more effective against the growth of microtumors.

Bergers *et al.* reported that TNP-470, angiostatin, BB-94 (batimastat), and endostatin each produced distinct efficacy profiles in a trial aimed at preventing the angiogenic switch in premalignant lesions, intervening in the rapid expansion of small tumors, or inducing the regression of large end-stage cancers.<sup>21</sup> They indicated that a 4-week treatment with TNP-470 caused significant regression of tumor burden and extension of life span in large end-stage cancer. Therefore, we attempted to prolong the treatment period (4 weeks) of FR-118487 in a late resection trial. Inhibitory effects of prolonged treatment on both hepatic and peritoneal metastases in the progressive stage of colon cancer were clearly demonstrated. Our results suggested that prolonged treatment with fumagillin derivatives might be needed after removal of a large tumor in the advanced stage of colon cancer.

Whenever primary tumors were removed, peritoneal metastases were prevented completely by the administration of FR-118487 after surgery. Because peritoneal metastases were not observed in the control group, the occurrence of peritoneal metastasis might be caused by the dissemination of tumor cells at cecal removal. This phenomenon also seems to mimic the clinical situation,

because surgical maneuvers sometimes implant tumor cells in the peritoneum; this is an important problem in surgery for colorectal or gastric cancer. Though additional studies should be performed to clarify the inhibitory mechanism of FR-118487 on peritoneal dissemination, the inhibition of neoangiogenesis at the peritoneal metastatic site may contribute to this effect. However, it is also possible that FR-118487 acts to decrease the activity of proteases.

The combination treatment of surgery and FR-118487 improved the survival, because untreated mice died within 60 days (data not shown), and the mice that underwent surgery alone died within 106 days after tumor inoculation due to metastasis. Thus, the additional administration of FR-118487 clearly improved the survival. Interestingly, half of the FR-treated mice were living at the end of the observation period. We reported that an angiogenesis inhibitor improved the long-term survival of rats with hepatic metastasis of rat carcinoma AH-130,<sup>22</sup> and metastatic liver tumors in rats treated with angiogenesis inhibitor decreased in size.<sup>23</sup> Because a weak ischemic stimulus is well known to trigger apoptosis<sup>24</sup> and it has been reported that angiogenesis inhibitors control metastatic growth by indirectly increasing apoptosis in tumor cells,<sup>25, 26</sup> we suggested that the angiogenesis inhibitor induced a weak ischemic state which triggered the apoptosis of tumor cells, causing the metastatic tumor to shrink. In the present study, two of the four mice, which were surviving at the end of the experiment, had no liver or peritoneal metastasis, and have continued to survive for 12 months.

The dominant adverse effect of FR-118487 treatment was body weight loss, which was also true of TNP-470. However, the 1-week infusion of FR-118487 did not induce a significant decrease of body weight gain, and in the "primary tumor removal" experiment, the body weights had recovered satisfactorily by the end of the experiment.

In conclusion, the administration of fumagillin derivatives followed by the resection of primary lesions may improve the prognosis of patients with colorectal cancer.

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