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Antitumor effect of the angiogenesis inhibitor TNP-470 on human digestive organ malignancy

Abstract The antitumor and antimetastatic effects of TNP-470, an angiogenesis inhibitor, on human gastrointestinal tumors xenotransplanted into nude mice were investigated. When two gastric cancer (MT-2 and MT-5) and two colon cancer (TK-4 and TK-13) xenografts are transplanted orthotopically into nude mice, liver metastasis develops 6 weeks after transplantation. TNP-470 30 mg/kg had a significant inhibitory effect on primary tumor growth of gastric cancers when given on alternate days from 7 days after transplantation. However, when given from 10 days or 14 days after transplantation, no inhibitory effect on the growth of any tumor xenograft was observed. In contrast, liver metastasis of each xenograft type was inhibited significantly by TNP-470. The effect of TNP-470 on prognosis was investigated using a hepatic metastatic model of rat hepatoma. Although all untreated rats that received AH-130 cell implants died within one month of massive hepatic metastasis, $>50\%$ of rats treated with TNP-470 survived for 4 months. The number of apoptotic cells in hepatic metastatic foci was significantly increased by TNP-470 administration. These results suggest that TNP-470 may provide a new approach to the treatment of digestive organ malignancies.

Key words Angiogenesis inhibitors · Hepatic metastasis · TNP-470

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Introduction

Antiangiogenic therapy is a new and potentially potent treatment for solid tumors, but was first proposed by Folkman in 1972 [6]. Since then, the mechanism of angiogenesis as well as novel angiogenic factors and angiogenesis inhibitors have been widely investigated.

Angiogenesis is controlled by various angiogenic factors and angiogenesis inhibitors [10]. When solid tumors grow, angiogenesis is "switched on" by an increase in angiogenic factors and/or a decrease in angiogenesis inhibitors [10]. Neoangiogenesis is also essential for tumor cells to form metastatic foci in the liver. Accordingly, administration of angiogenesis inhibitors may be a potent treatment for hepatic metastasis.

Angiogenesis inhibitors can be classified into two groups: angiogenic factor-specific and angiogenic factor-nonspecific inhibitors. Recently, antiangiogenic therapy with angiostatin or endostatin has attracted considerable interest because these representative angiogenic factor-nonspecific angiogenesis inhibitors have a strong antitumor effect which results in "tumor dormancy," as demonstrated by O'Reilly et al. [14, 15] and Boehm et al. [3].

Inhibition of hepatic metastasis can be expected to improve the prognosis of patients with digestive organ malignancies. However, the therapeutic effect of angiogenesis inhibitors on hepatic metastasis has not yet been demonstrated. Therefore the therapeutic effect of the angiogenic factor-nonspecific angiogenesis inhibitor TNP-470, which is an analogue of fumagillin, on hepatic metastasis of digestive organ malignancies was assessed.

Materials and methods

Reagents

TNP-470 (AGM-1470) was a gift from Takeda Chemical Industries, Osaka, Japan. It was suspended in vehicle consisting of 1%

ethanol plus 5% gum arabic in saline. The activity and characteristics of TNP-470 have been described elsewhere.

Animals

BALB/c-nu/nu mice were purchased from Clea Japan, Inc., Tokyo, Japan and male Donryu rats weighing 100–120 g were purchased from SLC, Inc., Hamamatsu, Japan. The experimental protocol followed the guidelines set out in the manual of Hamamatsu University School of Medicine.

Tumors

Xenografts were established from surgical specimens obtained from the Second Department of Surgery, Hamamatsu University School of Medicine and were maintained by subcutaneous passage in nude mice. Two human gastric cancer xenografts (MT-2 and MT-5), derived from poorly differentiated adenocarcinoma, and two colon cancer xenografts (TK-4 and TK-13) from well-differentiated adenocarcinoma were used.

The AH-130 cell line was donated by Kyowa Hakko Ltd., Fuji, Japan, and was maintained by serial intraperitoneal implantation in male Donryu rats. Ascites on day 10 of passage was detected by trypan blue dye exclusion.

Experimental design

Human xenografts

The method of orthotopic tumor transplantation was based upon that reported previously [8, 9]. In each experiment, mice were randomly divided into a control group and a treatment group on day 10 after transplantation. In the early treatment experiments, TNP-470 was administered on day 7 after transplantation. Mice in the treatment group were given TNP-470 30 mg/kg sc of vehicle on alternate days. The same volume of saline was administered intraperitoneally to mice in the control group. On day 42 after transplantation, all mice were weighed to assess body weight gain and killed to enable evaluation of primary tumor growth and macroscopic hepatic metastasis; specimens were also examined histologically.

Rat hepatoma

A rat hepatoma experiment was designed to evaluate the long-term therapeutic effect of TNP-470. The AH-130 cell line was a gift from Kyowa Hakko Ltd., Fuji, Japan. Cells (4×10^6) into rat superior mesenteric veins. In the control group, 10 rats received 0.6 mL NaCl solution (0.9%). The low-dose TNP-470 group (L-TNP; $n = 11$) was treated with TNP-470 15 mg/kg body weight sc every other day for a total of seven treatments. The first dose (injection volume 0.6 mL) was given 24 h after AH-130 implantation. The high-dose TNP-470 group (H-TNP; $n = 10$) was treated with TNP-470 30 mg/kg body weight sc every other day for a total of seven treatments. The first dose (injection volume 1.2 mL) was given 24 h after AH-130 implantation.

Treatment with TNP-470 was stopped on day 14. We confirmed the presence of metastatic foci on the surface of the liver after laparotomy, but metastatic foci were not counted. Animals were subsequently kept until 120 days after implantation of AH-130 cells.

Statistical analysis

Data on tumor weight and body weight are given as mean ± standard deviation. Tumor weight and body weight were analyzed for significance using the Student *t*-test. The χ^2 test and Fisher's exact test were used to compare the number of mice with hepatic metastasis; $P < 0.05$ was considered significant. Survival curves were calculated according to the Kaplan-Meier method and the differences between the groups were analyzed using the Mantel-Cox test.

Results

Inhibitory effect on primary tumor growth of human xenografts

The primary tumor implantation rate was 100% in all groups, and growing tumors were histologically confirmed to be composed mainly of cancer cells. TNP-470 administered from 10 days after tumor transplantation did not inhibit the growth of either gastric or colon cancer xenografts (Fig. 1). However, when TNP-470 was administered from day 7 after transplantation of MT-2 and MT-5 (early treatment), primary tumor growth was significantly inhibited ($P < 0.01$) (Fig. 2).

Inhibitory effect on hepatic metastasis of human xenografts

As shown in Table 1, all four tumor xenografts had high metastatic potential to the liver. However, TNP-470 significantly inhibited the hepatic metastasis of all tumors when assessed as a reduction in the number of mice with hepatic metastases.

Body weight gain

Early (day 7) TNP-470 administration was associated with a significant decrease in body weight gain of mice with MT-2 and MT-5 transplants (MT-2: 25.53 ± 2.05 g in the control group and 23.56 ± 1.91 g in the

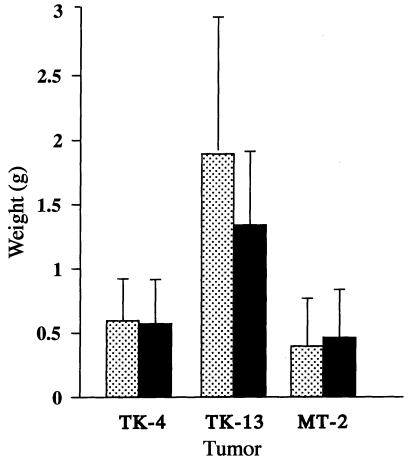

Fig. 1 Inhibitory effect of TNP-470 on tumor growth in nude mice when TNP-470 30 mg/kg was given on alternate days from 10 days after transplantation (▨ control group; ■ TNP-470 group). Results show the weight of the primary tumor xenotransplanted to orthotopic sites on day 42 after transplantation

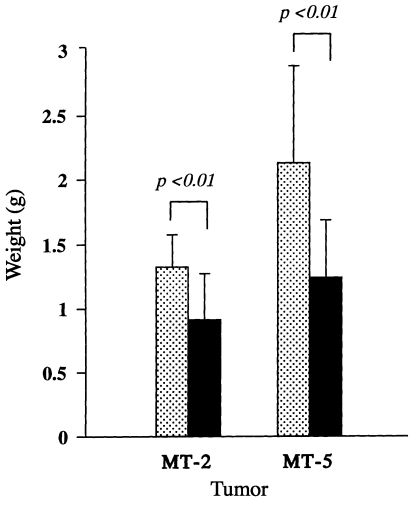

Fig. 2 Inhibitory effect of TNP-470 on gastric tumor growth in nude mice when TNP-470 30 mg/kg was given on alternate days from 7 days after transplantation (▨ control group; ■ TNP-470 group). Results show the weight of the primary tumor xenotransplanted to orthotopic sites on day 42 after transplantation

TNP-470-treated group, $P < 0.05$; MT-5: 24.65 ± 1.41 g in the control group and 20.57 ± 1.88 g in the TNP-470-treated group, $P < 0.001$).

Effect of TNP-470 on long-term survival

In the rat hepatoma experiment designed to evaluate the long-term effects of TNP-470, rats in the control group began to die 17 days after tumor implantation and all rats died due to massive liver metastasis within 28 days. Some of these rats developed ascites before death and became severely cachectic. On laparoscopy, all rats in the L-TNP and H-TNP groups had liver metastases after two weeks of TNP-470 treatment. In the L-TNP group, two of 11 rats died due to massive liver metastasis, one on day 21 and the other on day 25 after AH-130 cell implantation. In the H-TNP group, rats began to die on day 23 postimplantation and four rats had died by day 38 postimplantation due to intraperitoneal or intrapleural bleeding, which was regarded as an adverse effect of TNP-470. No histological injury to the liver, lung, heart, or kidney was demonstrated. Autopsy of all surviving rats (9 L-TNP + 6 H-TNP = 15) on day 120 postimplantation revealed no evidence of macroscopic metastatic foci; however, one dormant metastatic focus was found microscopically in one rat in the L-TNP group. The survival of rats in both the L-TNP and H-TNP groups was statistically significantly better than that of untreated controls ($P < 0.001$). Moreover, survival in the L-TNP group was significantly better than that in the H-TNP group ($P < 0.01$) (Fig. 3).

In a repeat experiment, all control rats died within one month after AH-130 cell implantation. The survival rate of L-TNP and H-TNP rats was 80% and 50%, respectively.

Table 1 Effect of TNP-470 on hepatic metastasis

Xenograft	Group	No. of mice with metastasis/total (%)
TK-4	Control	7/9 (77.8)
	Treatment	1/10 (10.0)[a]
TK-13	Control	11/11 (100.0)
	Treatment	4/10 (40.0)[a]
MT-2	Control	7/10 (70.0)
	Treatment	0/9 (0.0)[a]
MT-5	Control	8/10 (80)
	Treatment	2/11 (18.2)[b]

[a] $P < 0.01$ vs control group
[b] $P < 0.05$ vs control group

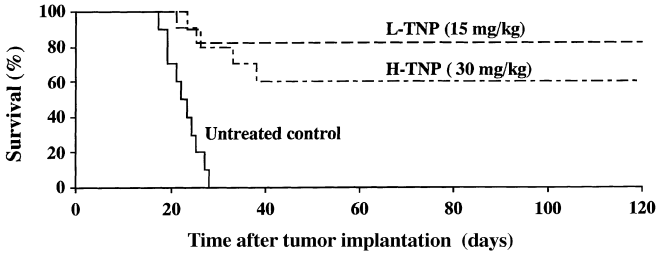

Fig. 3 Effect of TNP-470 on the survival of rats with liver metastasis produced after implantation of AH-130 cells. The survival of both L-THP and H-THP rats was significantly better than that of untreated controls ($P < 0.001$)

Body weight

In the rat hepatoma experiment, recovery of body weight four weeks after the discontinuation of TNP-470 administration was observed. The short- and long-term effects of TNP-470 on body weight are shown in Table 2.

Discussion

Hepatic metastasis is a critical problem for patients with gastrointestinal malignancies. About 30% of metachronous metastases of colorectal carcinomas that have been curatively resected are found in the liver [5]. Resection of these liver metastases has been proven to be beneficial, but <25% of patients who present with metastases are eligible for this form of treatment [2]. Many patients with hepatic metastases are unresectable because they

Table 2 Body weight changes in rats bearing AH-130 tumors after TNP-470 treatment

Group	Day 14	Day 42	Day 120
Control	180.2 ± 23.8	–	–
L-TNP	172.0 ± 17.3	338.3 ± 39	456.1 ± 50.4
H-TNP	143.1 ± 15.3[a]	326.7 ± 38.3	426.7 ± 27.3

[a] $P < 0.01$ vs control group

have other metastasis or multiple hepatic metastases. Therefore the prevention of micrometastases after resection of a primary tumor would improve survival.

Angiogenesis is believed to be essential for solid tumor growth beyond a few cubic millimeters [14]. Tumor neovascularization also gives cancer cells a route for hematogenous metastasis because new blood vessels in tumors are highly permeable and thus enable cancer cells to enter the circulation. However, even if cancer cells escape from the primary tumor to a secondary site, inhibition of angiogenesis might render the micrometastasis dormant. Therefore it is believed that antiangiogenesis agents may be clinically useful for the prevention of tumor progression.

We have established hepatic metastasis models of human colon and gastric carcinoma using orthotopic transplantation. These models are considered to reflect the process of hepatic metastasis observed clinically, and were therefore used to investigate the antimetastatic effect of TNP-470. TNP-470 has been reported to be highly effective against a wide variety of tumors and metastases [11, 17, 18], and exerts its effect primarily by preventing tumor neovascularization.

In the present study, TNP-470 showed significant suppression of primary tumor growth after early treatment of two gastric cancer xenografts, but not after late treatment. The author and colleagues have previously reported that TNP-470 has no significant effect on primary tumors transplanted orthotopically [13, 16]. These conflicting results may reflect the possibility that immature and/or rapidly growing tumors are more dependent on angiogenesis than mature and/or slowly growing tumors and that sensitivity to this agent may vary among tumors. Thus the inhibitory effect of TNP-470 on primary tumors might be related to the relative importance of angiogenesis at each stage of growth. Hepatic metastasis was also significantly prevented by TNP-470 administration, and this was independent of its effect on primary tumor growth. These findings suggest that TNP-470 is a potent antimetastatic agent.

By the time malignant tumors are diagnosed, many patients already have micrometastases which cannot be detected [4]. Thus administration of angiogenesis inhibitors such as TNP-470 may keep the micrometastases dormant, and coadministration of cytotoxic drugs might kill them. Kato et al. have demonstrated that combination therapy with standard cytotoxic agents enhances the antitumor effect of TNP-470 in an additive and dose-dependent manner [12].

The effect of TNP-470 on survival was clearly demonstrated in the AH-130 cell experiment. Interestingly, although liver metastases were confirmed in 95% (11/11 rats in the L-TNP group and 9/10 rats in the H-TNP group) of treated rats after TNP-470 administration for two weeks, only one dormant metastatic focus in one rat (L-TNP) was found after 120 days. Tumor cells in rats in the treatment groups at the end of TNP-470 administration were viable; therefore cell death was induced after treatment. It is suggested that sustained cytostatic inhibition of endothelial cells by TNP-470 is crucial in inducing later cell death. However, the mechanism of abrogation of metastatic nodules in TNP-470-treated rats is unknown, although, as discussed above, it is possible that TNP-470 induces apoptosis of tumor cells, presumably by the induction of a weak ischemic state. It has been observed that scattered apoptotic bodies are found in the livers of treated rats at significantly higher rates than in untreated control rats [1]. Therefore it is concluded that apoptosis may play a role in the regression of liver metastases. Complete understanding of the actions and interactions of the genetic, biochemical, and cellular mechanisms of apoptosis is essential to the development of therapies that can manipulate apoptosis for the treatment of cancer.

The loss of body weight induced by TNP-470 in the nude mouse has been reported previously [12, 13, 16]. However, recovery of body weight in surviving rats appears to occur within four weeks after the termination of TNP-470 administration according to the data presented here, suggesting that any body weight loss induced by TNP-470 is reversible. The intraabdominal and intrapleural bleeding observed four weeks after the termination of treatment in the H-TNP group is more important. Although the exact mechanism of bleeding is not clear, these results suggest that these adverse effects will appear only with long-term administration of TNP-470, which indicates that long-term observation is necessary for its safe clinical application. However, these fatal adverse effects were not observed in the L-TNP group and the survival rate in this group was higher than that in the H-TNP group; therefore the selection of the optimal dose for clinical use appears to be important.

In conclusion, although some problems remain to be solved, antiangiogenesis therapy may be a new strategy for treating hepatic metastasis of digestive organ malignancies.

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