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• LARGE INTESTINAL CANCER •

Angiogenesis inhibitor TNP-470 suppresses growth of peritoneal disseminating foci of human colon cancer line Lovo

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Abstract

AIM: To study the effect of angiogenesis inhibitor TNP-470 on peritoneal dissemination of colon cancer in nude mice.

METHODS: The MTT assay was used to evaluate the inhibitory effect of TNP-470 on human colon cancer cell line Lovo. Lovo cells were injected into the peritoneal cavity of BABL/C nu/nu mice and the models of peritoneal dissemination were developed. Thirty nude mice were randomly divided into control and TNP-470-treated group. In TNP-470-treated group, TNP-470 was injected subcutaneously every other day from day 1 until sacrifice or death (30 $mg \cdot kg^{-1}$). The control group received a sham injection of the same volume saline solution.

RESULTS: *In vitro*, TNP-470 inhibited the growth of Lovo cells, with its IC50 at 2.14×10^2 $\mu g \cdot L^{-1}$. *In vivo*, TNP-470 demonstrated growth inhibition of tumors. Mice body weight and abdominal circumferences were significantly different between TNP-470-treated group (24.5±3.2 g, 7.0±1.1 cm) and control group (29.5±2.1 g, 10.3±1.5 cm), P=0.005 and P=0.001. The number of disseminated foci was significantly different between the control group (92.1±20.6) and the TNP-470-treated group (40.3±12.3), P<0.001. The maximal size of foci was significantly smaller in TNP-470-treated group (3.3±0.7 mm) than that of control (7.3±2.3 mm), P=0.004. Mean survival time was significantly longer in TNP-470-treated group(98.00±12.06 d) than that in control group (41.86±9.51 d), P<0.001.

CONCLUSION: Angiogenesis inhibitor TNP-470 might be effective in treating peritoneal dissemination of colon cancer and improve the survival rate of nude mice.

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INTRODUCTION

Colorectal cancer still remains the most frequent malignancy in Japan, Unite States of American and China. Although combined therapies, including chemotherapy, radiation therapy and immunotherapy are performed in addition to surgical radical resection, nearly 50 % of patients still die of recurrence and a major form of recurrence was peritoneal dissemination[1]. Therefore, new therapeutic programs are needed to raise the survival rate of colorectal cancer patients. The importance of tumor angiogenesis is widely accepted in cases of blood-born metastases[2,3]. Although the form of blood supply is markedly different between metastases in solid organs and those at the peritoneum, it has been generally accepted that any foci larger than 0.2 mm require new tumor vessels for their growth[1-4]. Thus, inhibition of angiogenesis would prevent the tumor growth and their peritoneal dissemination.

TNP-470 is a semisynthetic analogue of fumagillin isolated from *Aspergillus fumigatus*. TNP-470 has been reported to inhibit neovascularization by preventing endothelial cells growth and proliferation[5-9]. Recently, the therapeutic effects of TNP-470 on various human and rodent tumors have been reported and this agent shows a marked inhibitory effect on tumor growth and metastasis *in vivo*[10-11]. However, the importance of angiogenesis in the establishment and growth of peritoneal dissemination remains unknown and there has been no report that evaluates the effect of TNP-470 on establishment and growth of peritoneal dissemination and ascites production of human colon cancer.

In this study, we investigated the inhibitory effects of TNP-470 on an establishment and growth of intraperitoneally inoculated human colon cancer cell line, Lovo, and survival of nude mice with this tumor *in vivo*. We also examined the inhibitory effect of TNP-470 on cell growth *in vitro*.

MATERIALS AND METHODS

Drug and reagents

TNP-470 was a generous gift from Takeda Chemical Industries (Osaka, Japan). 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), gum arabic and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO); RPMI 1640 and heat-inactivated fetal calf serum (FCS) were purchased from Gibco (Grand Island, NY).

TNP-470 was stored dry at -20 ℃. *in vitro* experiments, TNP-470 was dissolved in DMSO and RPMI 1640 medium supplemented with 10 % FCS. The final concentration of DMSO was 0.1 %, while *in vivo* experiments, TNP-470 was suspended in a vehicle of 3 % ethanol and 5 % gum arabic in saline.

Cell line

Human colon adenocarcinoma cell line, Lovo was kindly provided by the Department of Pathology, Cancer Center, First Military Medical University(FIMMU). Cells were cultured in RPMI 1640 supplemented with 10 % FCS, and were maintained at 37 ℃ in 5 % CO.All experiments were performed using cells harvested at the 80-90 % subconfluent stage.

Animals

Female BALB/c nude mice were obtained from the Experimental Animal Center, FIMMU, and reared under specific pathogene-free condition. Four-week-old mice weighing 17-22 g were used in the experiments.

In vitro experiments

The MTT assays were made to evaluate the sensitivity of TNP-470 to Lovo cells[8]. Lovo cells were plated in 96-well mocrotiter plates at a concentration of 5×10^4 cells in 50 μL of RPMI 1640 medium. After 24 h incubation, the medium was changed to RPMI 1640 medium with various concentrations of TNP-470(5×10^{-4} μg· L^{-1}-5×10^5 μg· L^{-1}), and the medium was incubated for 48 h, 20 μL MTT (5 g· L^{-1}) solution was then added to each well. After the plates were incubated for 3 h, 150 μL DMSO was added. The absorbance at 570 nm was determined using a microplate reader (Bio-Rad Model 3550, Hercules, CA). Dose-response curves were plotted, and the 50 % inhibitory concentration (IC50) was extrapolated as the drug dose causing a 50 % reduction in absorbance as compared with control values. The experiments were repeated in three independent experiments.

In vivo experiments

Lovo cells were harvested after being cultured for 48 h and the model of peritoneal dissemination in nude mice was developed as follows. Approximately 5×10^7 cells in 0.2 mL saline solution were injected into the peritoneal cavity of an nude mouse (day 0). Thirty nude mice were randomly divided into a control group (n=16) and a TNP-470-treated group (n=14). In the TNP-470-treated group,TNP-470 of 30 mg· kg^{-1} was injected subcutaneously every other day from day 1 until sacrifice. The control group received a sham injection of the same volume of saline. On day 10, two mice in control group were sacrificed and disseminated nodules on the peritoneum were evaluated. Seven mice each were sacrificed on day 30. Body weight and abdominal circumferences (substitute abdominal circumferences for ascites) of each mouse in two groups were measured. The number and maximum size of disseminated nodules on the peritoneum and mesentery were evaluated. The remaining 14 mice, 7 in each group, were followed for the survival experiment.

Statistical analysis

Data were expressed as mean ±standard deviation. Comparison between two groups was made by the independent samples t test. The survival curve was calculated by the Kaplan-meier method and compared by the Log-rank test. $P<0.05$ was considered statistically significant. All statistics were carried out using SPSS10.0 statistics software.

RESULTS

Effects of TNP-470 on cell growth in vitro

In the colorimetric MTT assay, significant growth inhibition was observed in a dose-dependent manner. The IC50 value was 2.14×10^2 μg· L^{-1} extrapolated from the dose-response curve following 48 h exposure (Figure 1).

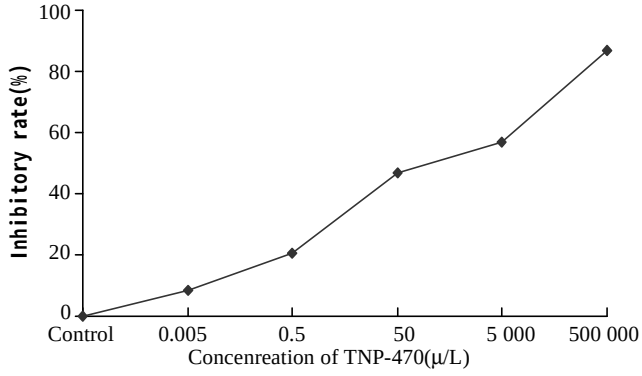

Figure 1 Inhibition curve of Lovo cells after TNP-470 treated 48 h

Effects of TNP-470 on establishment and growth of peritoneal dissemination in vivo

Two mice in control group sacrificed on day 10 developed disseminated nodules, suggesting that small nodules at the peritoneum developed within 10 days after inoculation in this model. Body weight and abdominal circumferences were gained in two groups when mice sacrificed on day 30. Body weight and abdominal circumferences in the control group and the TNP-470-treated group are summarized in Table 1. The difference of body weight and abdominal circumferences were statistically significant (P=0.005 and P=0.001). The number and maximum size of disseminated foci of the control group and the TNP-470-treated group are shown in Table 2. The difference of foci number and maximal size of disseminated foci were also statistically significant (P<0.001 and P=0.004).

Table 1 Body weight and abdominal circumference of two groups ($\bar{x}\pm s$)

Groups	Mice	Body weight (g)	Abdominal circumference (cm)
Control	7	29.5±2.1	10.3±1.5
TNP-470	7	24.5±3.2[a]	7.0±1.1[b]

[a]t=3.394, P=0.005, [b]t=4.624, P=0.001, *vs* control

Table 2 Numbers and Maximum size of disseminated foci of two groups ($\bar{x}\pm s$)

Groups	Mice	Number of foci	Maximum size of foci (mm)
Control	7	92.1±20.6	7.3±2.3
TNP-470	7	40.3±12.3[a]	3.3±0.7[b]

[a]t=5.715, P<0.001, [b]t=4.319, P=0.004, *vs* control

In the control group, mice died from day 31 to day 57. In a TNP-470-treated group, 5 mice were died from day 74 to day 110. Two mice survived more than 120 days and TNP-470 treatment was continued. These 2 mice did not have any disseminated foci and were sacrificed on day 120 and day 130, respectively. The median survival time in the control group and the TNP-470-treated group were 40 and 92 days, the mean survival time being 41.86±9.51 days and 98.00±12.06 days, respectively (P<0.001). The survival rate was significantly smaller in those in the control group than those of TNP-470-treated group (P<0.001) (Figure 2).

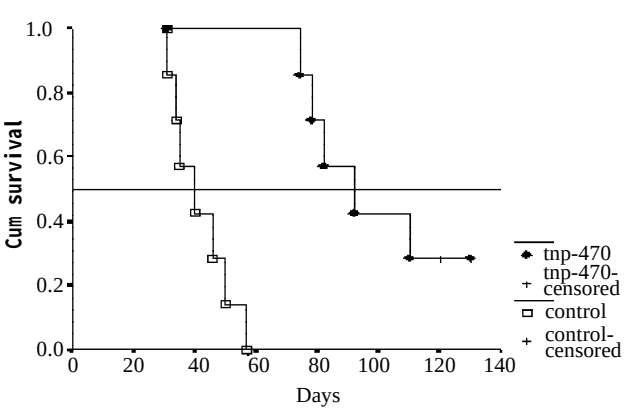

Figure 2 Survival curves in the control and TNP-470-treated groups

DISCUSSION

About 33 % patients with colorectal cancer have recurrence after operation and 50 % patients died of tumor metastasis[13,14], and the peritoneal dissemination or liver metastasis represents the most common form of recurrence. When the tumor has extended through the serosa or been resected , tumor cells are carried to distant points of the peritoneal cavity and "seeding" on peritoneum. Supported by peritoneal permeability and growth of neovascularization, these tumor cells would develop into micro-metastatic nodules, eventually producing generalized peritoneal dissemination[15,16]. With tumor recurrence as peritoneal dissemination, patient prognoses are extremely poor. Although combined therapies, including chemotherapy, radiation therapy and immunotherapy are performed in addition to surgical radical resection, no effective treatment can prevent the recurrence. New therapeutic strategies have to be invented to overcome the poor prognosis.

Angiogenesis, has been shown to be essential for tumor growth not only at the primary but also at the site of metastases, and the peritoneum would not be an exception[17-20]. Inhibition of angiogenesis is emerging as a promising strategy for cancer treatment[21-24]. Anti-angiogenic agents have demonstrated a remarkable inhibition effect on tumor growth and metastasis, and anti-angiogenic therapy may prevent the tumor recurrence[25].

Among the most potent inhibitors of angiogenesis is the fumagillin family of natural products. An analog of fumagillin, known as TNP-470 or AGM-1470, has the anti-angiogenic activity by inhibiting endothelial cell growth with high potency both *in vitro* and *in vivo*[26,27]. Studies have shown that the molecular mechanism of TNP-470 inhibiting endothelial cell proliferation is associated with the two type methionine aminopeptidase (MetAp-2). TNP-470 was found to bind MetAp-2 covalently, leading to specific inhibition of its activity, and strong correlation has been found between inhibition of MetAp-2 enzymatic activity and inhibition of endothelial cell proliferation[28-33]. Investigators for TNP-470 have demonstrated suppression of neovascularization, tumor growth, and distant metastases *in vivo*[34-44], and the proliferation of various cancer cell lines *in vitro*[45-49]. There are very few studies on peritoneal dissemination[50-52], and no data are available about preventing peritoneal dissemination or survival benefit of colon cancer treated by TNP-470.

Tsujimoto *et al*[53] reported that angiogenesis occurred in peritoneal foci 1 week after intraperitoneal inoculation of tumor cells in a mouse model. Our study indicated that TNP-470 inhibied the proliferation of Lovo cells *in vitro*, with its IC50 at 2.14×10^2 ug/L, and markedly suppressed the growth of peritoneal dissemination in vivo. The mice survival time was siginificantly longer in TNP-470 treated group than that of control. Our results suggest that these effects are exerted not only by inhibiting neovascularization necessary for tumor growth, but also by directly inhibiting the proliferantion of Lovo cells.

Body weight loss was known to be the major side effect of TNP-470. There was a slight body weight gain when mice were sacrificed on day 30, and the increase was associated with the production of malignant ascites. In the survival experiments, body weight loss was observed in the control group and 3 tumor-bearing mice became cachectic with the progression of tumor. No body weight loss was observed in the treatment group, suggesting that suppression of the growth of peritoneal dissemination foci by TNP-470 resulted in a preservation of body weight and prevention of cachexia in this model.

In conclusion, angiogenesis inhibitor TNP-470 might be effective in treating peritoneal dissemination of colon cancer by inhibiting the growth of the seeded tumor cells on the peritoneum.

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