

EFFECT OF ANGIOGENESIS INHIBITOR TNP-470 ON THE PROGRESSION OF HUMAN GASTRIC CANCER XENOTRANSPLANTED INTO NUDE MICE

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The effect of an angiogenesis inhibitor, TNP-470, on primary tumor growth, liver metastasis and peritoneal dissemination of gastric cancer was investigated by means of an orthotopic xenotransplanted model of 2 human gastric cancers, MT-2 and MT-5. TNP-470 showed a significant inhibitory effect on the growth of primary tumors after orthotopic transplantation of both xenografts when given at a dose of 30 mg/kg on alternate days from day 7 after transplantation (early treatment). However, growth of the MT-2 primary tumor was not inhibited by administration from day 14 after transplantation (late treatment). Liver metastasis was prevented significantly by early treatment of TNP-470. In particular, early treatment of MT-2 completely inhibited the development of macroscopic foci in the liver and was significantly more effective than late treatment. Peritoneal dissemination also was inhibited. Thus, TNP-470 was revealed to have strong inhibitory activity not only on primary tumors and liver metastases but also against peritoneal dissemination. These results suggest that this agent may provide a new approach to the treatment of gastric cancer. *Int. J. Cancer* 71:838–841, 1997.

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Although the prognosis of gastric cancer has improved due to developments in diagnosis and treatment, liver metastasis and peritoneal dissemination, which are the predominant types of recurrence, are crucial problems after curative surgery. All patients with recurrence have micrometastases which cannot be detected at the time of diagnosis (Fidler and Balch, 1987). To further improve the prognosis, it is essential to eliminate these micrometastases. A preventive effect of chemotherapy on metastasis after curative resection has been reported for colorectal cancer. However, post-operative chemotherapy is not so effective for liver metastasis or peritoneal dissemination. Furthermore, treatment of recurrent gastric cancer has failed to improve the prognosis. Thus, a new approach to treatment and prevention of the recurrence of gastric cancer needs to be established.

Tumors are always dependent on the development of an adequate blood supply through angiogenesis for growth at both primary and secondary sites. The concept of anti-angiogenesis therapy was first proposed in 1971 (Folkman, 1971, 1985). The implications of angiogenesis for tumor biology and therapy have been investigated, and some anti-angiogenesis agents have been developed. TNP-470 is a potent angiogenesis inhibitor and an analogue of fumagillin, which is a natural antibiotic secreted by *Aspergillus fumigatus* fresenius (Ingber *et al.*, 1990; Kusaka *et al.*, 1991). This agent shows a marked inhibitory effect on tumor growth and metastasis *in vivo* (Ingber *et al.*, 1990; Konno *et al.*, 1995; Tanaka *et al.*, 1995; Yanase *et al.*, 1993). Its main target is not the cancer cells themselves but the endothelial cells of the host, and the tumor is affected indirectly (Ingber *et al.*, 1990; Kusaka *et al.*, 1991). Thus, there are no problems related to the heterogeneity of cancer cells or to the emergence of resistance.

The process of cancer metastasis consists of a complicated sequence of steps (Poste and Fidler, 1980). In an orthotopic transplantation model of human cancer, tumor cells must escape from the primary lesion and complete all of the events in the metastatic cascade before establishing secondary foci. This model allows the properties of transplanted tumors to be retained by the insertion of intact tissue from humans into nude mice (Fu *et al.*, 1991; Furukawa *et al.*, 1993). Accordingly, results obtained in this model are thought to be of relevance to the clinical situation.

In the present study, we examined the inhibitory effect of TNP-470 on tumor growth and metastasis of human gastric cancer after orthotopic xenotransplantation into nude mice.

MATERIAL AND METHODS

Drugs

TNP-470 was kindly donated by Takeda Chemical Industries (Osaka, Japan). Its structure and characteristics have been reported (Ingber *et al.*, 1990; Kusaka *et al.*, 1991). TNP-470 was suspended in a vehicle of 1% ethanol plus 5% gum arabic in saline.

Animals

Male BALB/c-*nu/nu* mice were obtained from Clea (Tokyo, Japan). Five-week-old mice weighing 20 g were used in the experiments.

Human gastric-cancer xenografts

Two human gastric-cancer xenografts, MT-2 and MT-5, were used in this study. They were poorly differentiated adenocarcinoma lines, which were established and maintained by serial transplantation into nude mice in our department.

Orthotopic tumor transplantation

Tumors in the exponential growth phase were resected aseptically from nude mice, and healthy tumor tissue was minced into pieces about 5 mm in diameter. Mice were anesthetized with ether, and a midline incision was made in the upper abdomen. The stomach wall was carefully exposed and the serosa on the middle of the greater curvature was removed. A piece of tumor tissue was fixed to the site with a transmural suture of 6-0-coated Vicryl (Ethicon, Somerville, NJ), then the stomach was returned to the peritoneal cavity, and the abdominal wall and skin were closed with 6-0-coated Vicryl.

Experimental protocol

In each experiment, using MT-2 or MT-5 tumors, mice were assigned to a treated (TNP) group or a control group. In the TNP group, 9 or 11 mice received s.c. injections of TNP-470 at a dose of 30 mg/kg on alternate days from day 7 after tumor transplantation (early treatment). In each control group, 10 mice received s.c. physiological saline injections of the same volume.

In an additional experiment using MT-2, 7 mice received TNP-470 from day 14 after tumor transplantation (late treatment) and 10 mice received s.c. saline injections.

On day 42 after transplantation, mice were killed and weighed and autopsy was done carefully. Macroscopic liver metastasis and peritoneal dissemination were evaluated (Fig. 1). In addition, tumors growing on the stomach were removed, weighed and examined histologically in the usual manner.

Statistical analysis

The data of tumor weight and body weight are given as the mean \pm standard deviation and were analyzed for significance by

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FIGURE 1 – Macroscopic appearance of tumor growth and progression of human gastric cancer xenotransplanted orthotopically into nude mice (primary tumor, arrow; liver metastasis, arrowheads; peritoneal dissemination, *).

Student's *t*-test. The χ^2 and Fisher's exact tests were used to compare the number of mice with liver metastasis and peritoneal dissemination in each group, and $p < 0.05$ was considered significant.

RESULTS

Primary tumor growth

The take rate of the primary tumors (xenotransplanted to the stomach) was 100% in all groups, and growing tumors were histologically confirmed to be mainly composed of cancer cells (Fig. 2). By administration of TNP-470 from day 7 after transplantation (early treatment) of both MT-2 and MT-5, primary tumor growth was significantly inhibited (Table I). However, TNP-470 did not inhibit the growth of MT-2 when administered from day 14 (late treatment).

Liver metastasis

In early treatment of both MT-2 and MT-5, TNP-470 showed a significant inhibitory effect on liver metastasis (Fig. 3, Table II). In late treatment of MT-2, the percentage of mice with liver metastasis was decreased by administration of TNP-470, but the effect was not statistically significant. However, comparison of the number of metastatic foci showed a significant decrease even in the late treatment (51.6 ± 31.15 in the control group vs. 13.29 ± 14.89 in the TNP group, $p < 0.05$). In particular, early treatment of MT-2

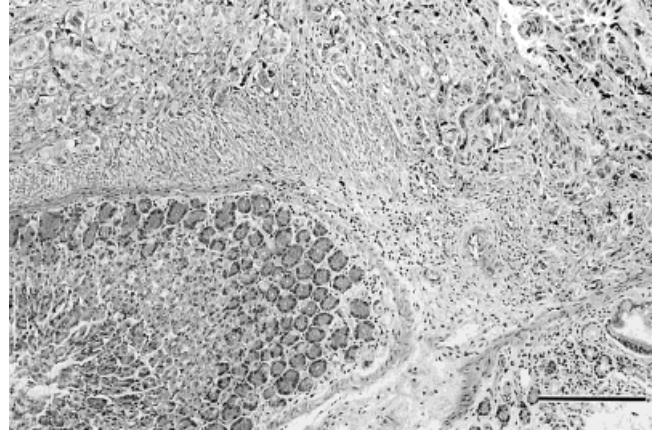


FIGURE 2 – Histological appearance of primary tumor xenotransplanted on the stomach. Poorly differentiated adenocarcinoma invaded to the muscular layer of the stomach. Scale bar: 200 μ m.

TABLE I – INHIBITORY EFFECT OF TNP-470 ON PRIMARY TUMOR

	Xenograft	Primary tumor weight (g)
Early treatment		
Control group	MT-2	1.31 ± 0.24
TNP group	MT-2	0.87 ± 0.37^1
Control group	MT-5	2.12 ± 0.76
TNP group	MT-5	1.18 ± 0.52^2
Late treatment		
Control group	MT-2	0.42 ± 0.34
TNP group	MT-2	0.48 ± 0.40^3

¹ $p < 0.01$ vs. control group.–² $p < 0.01$ vs. control group.–³Not significant vs. control group.

completely inhibited liver metastasis and was significantly more effective than late treatment ($p < 0.05$).

Peritoneal dissemination

With late treatment of MT-2, macroscopic nodules of peritoneal dissemination appeared in 60% (6/10) of the mice in the control group. In contrast, no dissemination was seen in any mice from the TNP group. In the experiment using MT-5, 4 of 11 mice had dissemination in the TNP group compared with 7 of 10 in the control group (Fig. 4, Table III).

Body weight

In both experiments, early treatment caused a significant decrease of weight gain on day 42 after transplantation (MT-2: 25.53 ± 2.05 g in the control group and 23.56 ± 1.91 g in the TNP group, $p < 0.05$; MT-5: 24.65 ± 1.41 g in the control group and 20.57 ± 1.88 g in the TNP group, $p < 0.001$). However, this did not occur with late treatment of MT-2 (18.63 ± 2.44 g in the control group and 18.13 ± 1.44 g in the TNP group).

DISCUSSION

TNP-470 has been reported to be highly effective against a wide variety of tumors and metastases (Ingber *et al.*, 1990; Konno *et al.*, 1995; Tanaka *et al.*, 1995; Yanase *et al.*, 1993). This agent mainly exerts its anti-tumor effect by preventing tumor neovascularization (Ingber *et al.*, 1990; Kusaka *et al.*, 1991). Angiogenesis is essential for the growth of solid tumors at primary and at secondary sites (Folkman, 1971, 1985). It is thought that the new blood vessels in tumors are highly permeable (Dvorak *et al.*, 1988) and provide a route for cancer cells to enter the circulation (Folkman, 1971). However, even if cancer cells escape from the primary tumor to a secondary site, inhibition of angiogenesis might keep micrometas-

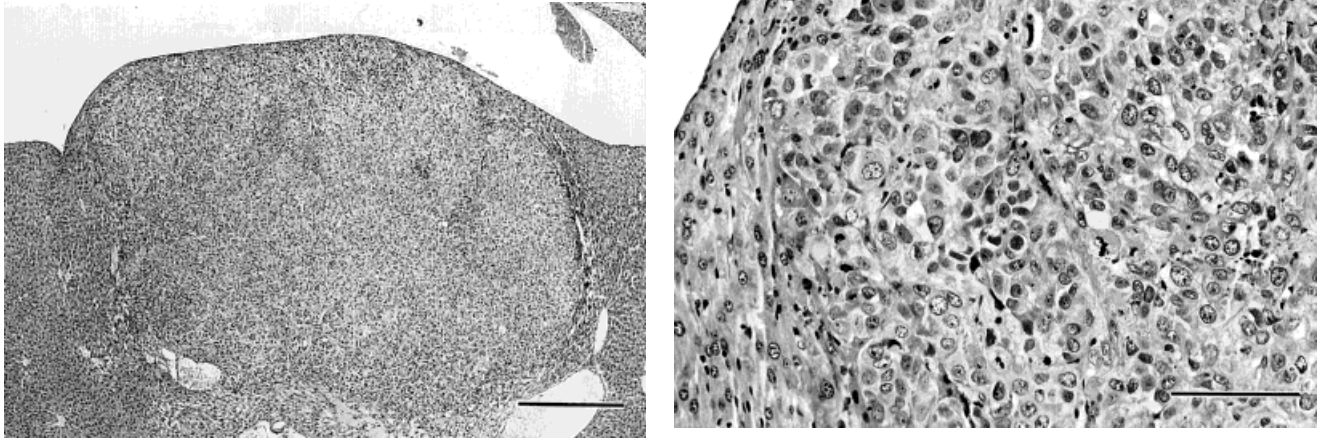


FIGURE 3 – Histological appearance of liver metastasis. Capsulated poorly differentiated adenocarcinoma was observed in the liver. (left panel) Low magnification; scale bar: 500 μm ; (right panel) high magnification; scale bar: 100 μm .

TABLE II – INHIBITORY EFFECT OF TNP-470 ON LIVER METASTASIS

	Xenograft	Number of mice with liver metastasis
Early treatment		
Control group	MT-2	7/10
TNP group		0/9 ^{1,4}
Control group	MT-5	8/10
TNP group		2/11 ²
Late treatment		
Control group	MT-2	10/10
TNP group		4/7 ³

¹ $p < 0.01$ vs. control group. ² $p < 0.05$ vs. control group. ³Not significant vs. control group. ⁴ $p < 0.05$ vs. TNP group with late treatment.

tases dormant (Folkman, 1985). Therefore, anti-angiogenesis agents may have the potential to be clinically useful for the prevention of cancer progression.

In the present study, an orthotopic xenotransplantation model with human gastric cancer was used. To produce metastasis, tumor cells must complete all of the steps, including angiogenesis, migration, invasion, survival in the circulation, adhesion, extravasation and proliferation (Poste and Fidler, 1980). Thus, orthotopic transplantation models are thought to closely resemble the clinical setting and to be useful for the development of new treatment modalities (Fu *et al.*, 1991; Furukawa *et al.*, 1993). Few cell lines reported show peritoneal dissemination. In this model, MT-2 and MT-5 have a metastatic potential for peritoneal dissemination as well as liver metastasis.

In the present study, TNP-470 showed significant suppression of primary tumor growth with early treatment of both gastric-cancer xenografts but did not do so with late treatment. Some reports have indicated that this agent has a potent inhibitory effect on tumors transplanted s.c. (Ingber *et al.*, 1990; Yanase *et al.*, 1993). In contrast, there are reports that it has no significant effect on primary tumors transplanted orthotopically (Konno *et al.*, 1995; Tanaka *et al.*, 1995). These differences may arise because of dependency on angiogenesis (Kim *et al.*, 1993). Thus, for the tumors used in the present study, angiogenesis may be more dominant in the early phase of growth than in the late phase. In addition, sensitivity to TNP-470 may vary among tumors (Yanase *et al.*, 1993). There have been reports of organ-site-dependent differences in tumorigenesis and in cancer-cell properties, such as production of b-FGF or degradative enzymes (Singh *et al.*, 1994) and sensitivity to cytotoxic drugs (Wilmanns *et al.*, 1992). However, to clarify the mechanisms involved, additional experiments need to be performed.

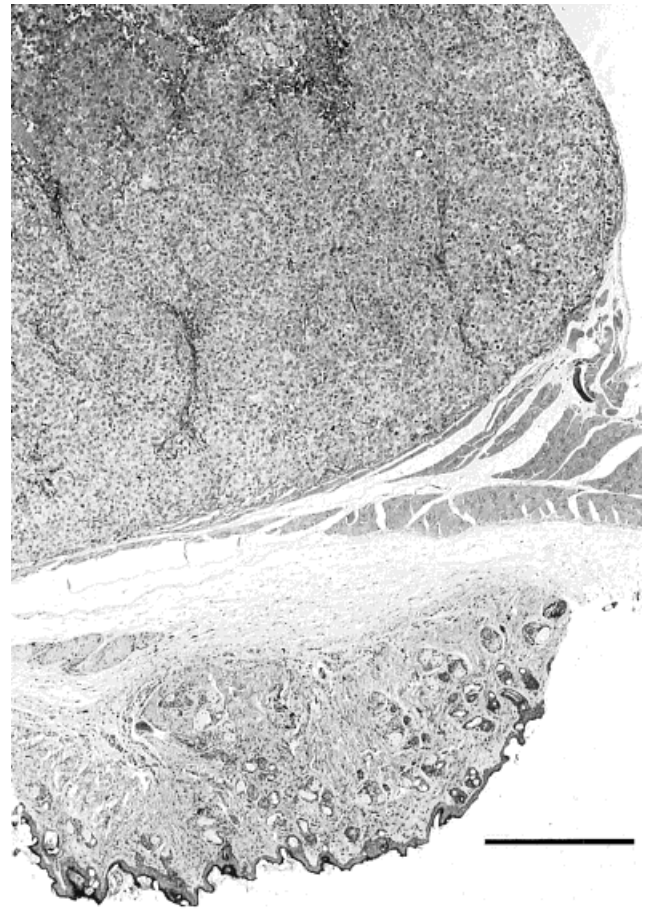


FIGURE 4 – Histological appearance of peritoneal dissemination. Poorly differentiated adenocarcinoma was disseminated in the peritoneum. Scale bar: 500 μm .

Liver metastasis was significantly inhibited by early administration of TNP-470 from day 7 after tumor transplantation. Late administration from day 14 decreased the percentage of mice with liver metastasis and had a significant inhibitory effect on the number of metastatic foci, but early treatment was much more effective than late treatment. It is unknown whether this was related

TABLE III - INHIBITORY EFFECT OF TNP-470 ON PERITONEAL DISSEMINATION

Xenograft		Number of mice with peritoneal dissemination
Early treatment		
Control group	MT-5	7/10
TNP group		4/11 ¹
Late treatment		
Control group	MT-2	6/10
TNP group		0/7 ²

¹*p* = 0.270 vs. control group. ²*p* < 0.05 vs. control group.

to an effect on the primary tumor or not, but our findings demonstrate that this agent may be a potent anti-metastatic drug. Further investigations on the optimal timing of treatment may help to clarify its clinical potential.

Kuo *et al.* (1993) studied orthotopically transplanted human colon cancer and found that cancer cells entered the circulation from the primary tumor from day 10 after transplantation. Koop *et al.* (1995) indicated that more than 80% of i.v. injected B16F10 melanoma cells survived and showed extravasation after 24 hr, suggesting that prevention of tumor growth after extravasation may be the key to controlling metastasis. It is possible that early inhibition of neovascularization at the primary site may prevent escape of tumor cells into the circulation and that blocking angiogenesis may keep tumor cells dormant, resulting in the inhibition of macroscopic metastasis. By the time malignant tumors are diagnosed, many patients already have micrometastases which cannot be detected (Fidler and Balch, 1987). Thus, administration of angiogenesis inhibitors such as TNP-470 may keep micrometastases dormant and co-administration of cytotoxic drugs might kill them. In this context, Kato *et al.* (1994) have demonstrated that combination therapy with standard cytotoxic agents

enhanced the anti-tumor effect of TNP-470 in an additive and dose-dependent manner.

Free cancer cells have been detected in the peritoneal cavity of some patients with gastric cancer, and their prognosis is poor. Generally, patients who have gastric cancer with serosal invasion are regarded as one of the highest risk groups for tumor cells being scattered from the primary site into the peritoneal cavity (Boku *et al.*, 1990). Some of these cancer cells may produce viable metastases. Established peritoneal dissemination is incurable, largely owing to the ineffectiveness of current chemotherapeutic agents.

Some studies have examined the effect of angiogenesis inhibitors on carcinomatous peritonitis caused by i.p. tumor inoculation. Tsujimoto *et al.* (1995) reported that i.p. administration of TNP-470 from day 1 after tumor inoculation extended survival time (angiogenesis occurs in peritoneal foci by 1 week after inoculation). Tumor cells released into the peritoneal cavity also require angiogenesis to grow into foci larger than a few cubic millimeters (Folkman, 1985). Therefore, TNP-470 may have an inhibitory effect on peritoneal dissemination by blocking neovascularization in the primary tumor and/or at sites of dissemination. In the present experiment, peritoneal dissemination was inhibited, but the mechanism is not clear. However, this finding suggests that TNP-470 also could be useful for preventing peritoneal dissemination.

Optimal anti-angiogenesis therapy is likely to require long-term administration of inhibitors with low toxicity. We found a decrease of weight gain after early treatment with TNP-470, regardless of the inhibition of tumor progression. However, we found that constant recovery of body weight after treatment was observed in the long-term experiment using rats (Ahmed *et al.*, 1996).

In conclusion, the angiogenesis inhibitor TNP-470 is a potential new therapy for gastric cancer, which may not only control the primary tumor and hematogenous metastases but may also inhibit peritoneal dissemination.

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