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Inhibition of Liver Metastasis of Human Gastric Carcinoma by Angiogenesis Inhibitor TNP-470

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The anti-tumor and anti-metastatic effects of TNP-470, an angiogenesis inhibitor, and mitomycin C (MMC), a representative anti-neoplastic agent, were investigated using our established liver-metastasizing gastric carcinoma line, AZ-H5c. AZ-H5c was injected into the spleen of nude mice which had been randomly divided into 4 groups; a control group given saline solution, a group receiving 15 mg/kg TNP-470, a group receiving 30 mg/kg TNP-470 and a group receiving 2 mg/kg MMC. TNP-470 was given s.c. on alternate days for 5 weeks from day 10 after intrasplenic injection, and MMC was administered intraperitoneally (i.p.) once a week from day 10 after intrasplenic injection. In the control group, liver metastasis developed in 13 of 16 mice (81%). Liver metastasis developed in 6 of 11 mice (55%) receiving MMC. In contrast, liver metastasis developed in 4 of 8 mice (50%) receiving 15 mg/kg TNP-470, and in 0 of 14 mice (0%) receiving 30 mg/kg TNP-470. However, TNP-470 had no effect on the tumor growth. These results indicate that the angiogenesis inhibitor TNP-470 has a strong inhibitory activity against *in vivo* liver metastasis of human gastric carcinoma.

Key words: Metastasis — Human gastric carcinoma — TNP-470 — Angiogenesis

Liver metastasis is very often observed in human gastric carcinoma, which is one of the most frequent causes of cancer death in Japan. To improve the cure rate of gastric carcinoma, more attention should be directed to early detection and prevention of metastasis. The establishment of relevant animal metastatic models of gastric carcinoma is highly important in the search for new therapeutics for gastric carcinoma. We have established cell lines, designated AZ-H1c, AZ-H2c, AZ-H3c,[1] AZ-H4c and AZ-H5c,[2] with various metastatic potentials in the liver by using intrasplenic injections of gastric carcinoma line AZ521.

Angiogenesis is essential for the growth of solid tumors,[3] especially in the early phase. This suggests that the process of tumor metastasis, especially the growth of metastatic cells, requires angiogenesis at the metastatic site and that inhibition of angiogenesis may be an effective treatment for metastasis of solid tumors. TNP-470 inhibited angiogenesis more potently than its parent compound fumagillin, both *in vivo* and *in vitro*, regardless of the presence of angiogenesis factors and the model of administration, and it was also less toxic than fumagillin.[4] In addition, it has been reported that TNP-470 has an inhibitory effect on the growth and metastasis of human cell lines[5–7] and rodent tumors.[8] In this paper, AZ-H5c, which has the highest metastatic potential, was used to compare the liver metastasis-inhibitory effect of TNP-470 with that of mitomycin C (MMC), a representative anti-neoplastic agent for gastric carcinoma.

MATERIALS AND METHODS

Chemicals TNP-470 was the kind gift of Takeda Chemical Industries, Ltd. (Osaka). Its structure, disposition and metabolism have already been reported.[9,10] TNP-470 was suspended in a vehicle composed of 0.5% ethanol plus 5% gum arabic in saline. MMC was dissolved in saline.

Animals Female BALB/c *nu/nu* mice, which had originated from the Central Institute for Experimental Animals (Kawasaki), were obtained from CLEA Japan, Inc. (Tokyo). Animals used were 6–7 weeks old and weighed 18–20 g.

Cell line A human gastric carcinoma cell line with high liver metastatic capability, AZ-H5c, was established by *in vivo* stepwise selections according to the method described by Morikawa *et al.*[11] in our laboratory. Briefly, a human gastric carcinoma line, AZ521 was injected into the spleen of nude mice. Cells from the few liver metastatic foci of injected AZ521 were expanded *in vitro* and subsequently injected into the spleen of nude mice. By repeating these procedures five times, we were able to obtain a cell line, designated AZ-H5c, with high metastatic potential in nude mice.

Assay of tumor growth *in vivo* Cultured AZ-H5c cells (5×10^6/0.1 ml in phosphate-buffered saline) were inoculated subcutaneously (s.c.) into nude mice. Mice were divided into 2 groups; a control group given saline solution ($n=5$) and a group receiving 30 mg/kg TNP-470 (n

=6). Then 0.1 ml of TNP-470 (30 mg/kg) was given s.c. on alternate days for 5 weeks from day 10 after s.c. injection. The resulting tumors were measured with calipers and the tumor volume was estimated by using the following formula; $V=a\times b^2/2$ (V, volume; a, the longest diameter; b, the shortest diameter).

Assay of liver metastasis The ability to form metastatic foci in the liver was determined following an intrasplenic injection as described by Morikawa *et al.*[11] Briefly, AZ-H5c cells (5×10^6/0.1 ml in phosphate-buffered saline) were injected into the spleen of nude mice using a 26-gauge needle. Mice were divided into 4 groups; a control group given saline solution ($n=16$), a group receiving 15 mg/kg TNP-470 ($n=8$), a group receiving 30 mg/kg TNP-470 ($n=14$) and a group receiving 2 mg/kg MMC ($n=11$). Then 0.1 ml of TNP-470 (15 or 30 mg/kg) was given s.c. on alternate days for 5 weeks from day 10 after intrasplenic injection and 0.1 ml of MMC (2 mg/kg) was administered i.p. once a week from day 10 after the intrasplenic injection.[12] The mice were killed approximately 5 weeks after the injection, and autopsy was performed. The liver was processed for routine histological examination to detect metastases after careful macroscopic examination. The resulting splenic tumors were measured with calipers and tumor volume was estimated by using the following formula; $V=a\times b^2/2$ (V, volume; a, the longest diameter; b, the shortest diameter).

Statistical analysis The unpaired t test was used for comparison of splenic tumor growth, subcutaneous tumor growth and body weight. The χ^2 test was used to compare the number of mice with liver metastasis in each group.

RESULTS

Tumor growth To evaluate the inhibitory effect of TNP-470 on the tumor growth, AZ-H5c cells were inoculated s.c. and intrasplenically into nude mice. Fig. 1 shows the subcutaneous tumor volume in the control group and the 30 mg/kg TNP-470 group. TNP-470 tended to inhibit

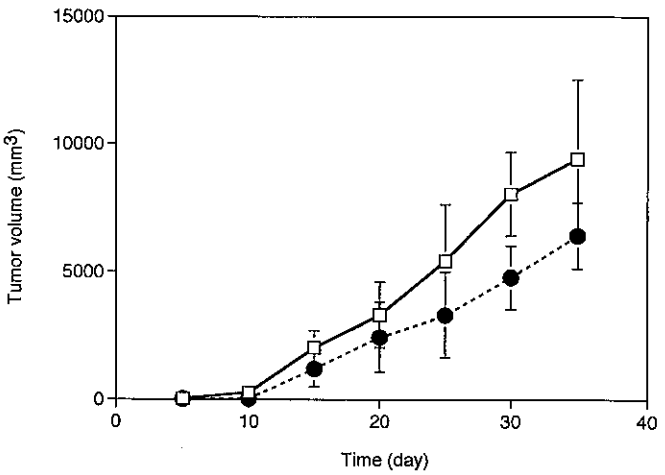

Fig. 1. Subcutaneous tumor growth in volume. Bars represent mean±standard error. □ control, ● TNP-470 (30 mg/kg).

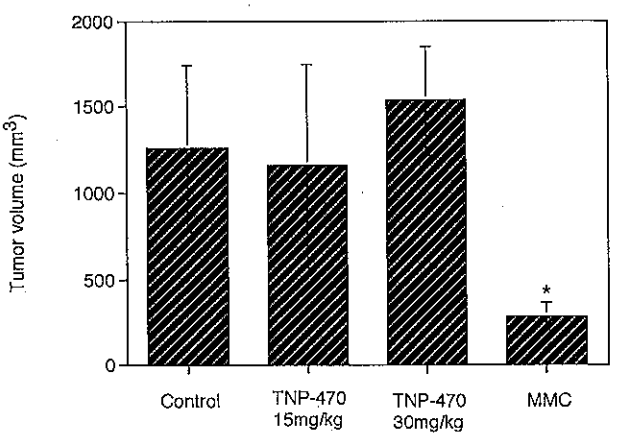

Fig. 2. Splenic tumor growth. Bars represent mean±standard error. * Statistically significant difference from the control group ($P<0.05$).

Table I. Inhibitory Effect of TNP-470 on Liver Metastasis

Group	Number of mice with liver metastasis		
	Experiment 1	Experiment 2	Total
Control	10/12 (83%)	3/4 (75%)	13/16 (81%)
TNP-470 15 mg/kg	4/8 (50%)	ND	4/8 (50%)
TNP-470 30 mg/kg	0/8 (0%)	0/6 (0%)	0/14 (0%)*
MMC	6/11 (55%)	ND	6/11 (55%)

ND: not determined.
* Statistically significant difference from the control ($P<0.01$).

Table II. Number of Metastatic Liver Foci

Group	Number of mice	0	0<foci<5	5<foci<10	10<
Control	16	3/16	11/16	0/16	2/16
TNP-470 15 mg/kg	8	4/ 8	2/ 8	2/ 8	0/ 8
TNP-470 30 mg/kg	14	14/14	0/14	0/14	0/14
MMC	11	5/11	6/11	0/11	0/11

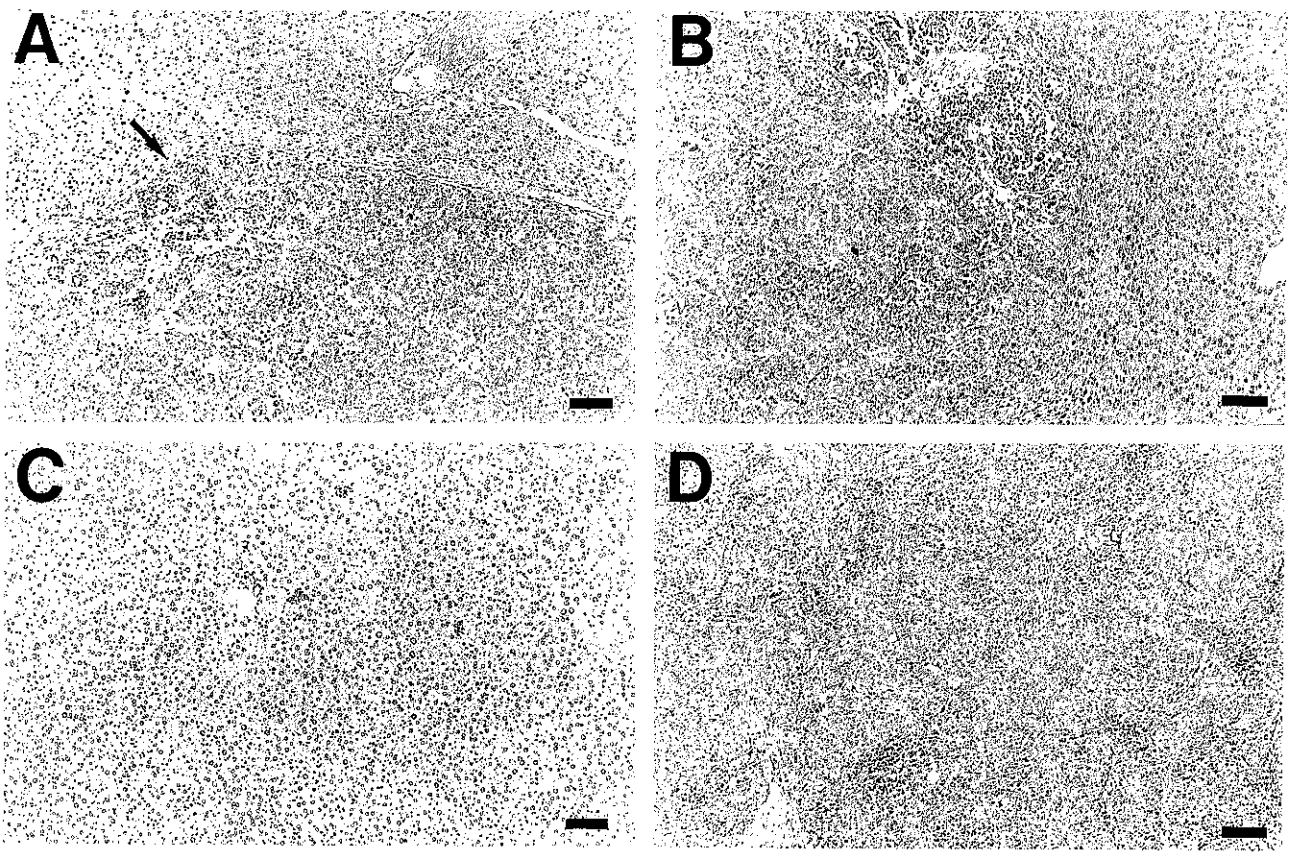

Fig. 3. Microscopic views of metastatic liver tumor. A, Control group; B, 15 mg/kg TNP-470 group; C, 30 mg/kg TNP-470 group; D, MMC group. Massive metastasis (arrow) was observed in the control group and MMC group, but not in the 30 mg/kg TNP-470 group. Tissues were fixed, embedded, sectioned, and stained with hematoxylin and eosin using standard procedures. ×50. Scale bar = 100 μm.

the subcutaneous tumor growth although the inhibition was not statistically significant.

Fig. 2 shows the splenic tumor volume at killing. The actual tumor volume was 1259±481 mm^3 in the control group, 1158±587 mm^3 in the 15 mg/kg TNP-470 group, 1534±317 mm^3 in the 30 mg/kg TNP-470 group and 283 ±83 in the MMC group. TNP-470 had no inhibitory effect on splenic tumors, but MMC inhibited splenic tumor growth ($P=0.034$).

Liver metastasis Table I summarizes the effects of TNP-470 and MMC on liver metastasis. Administration of 30 mg/kg TNP-470 completely inhibited liver metastasis macroscopically and microscopically. Liver metastasis developed in 4 of 8 mice (50%) receiving 15mg/kg TNP-470. In contrast, the anti-metastatic effect of MMC was not as strong as that of TNP-470, although the number of metastatic foci was lower in the MMC group than in the control group (Table II). All the metastatic lesions were

confirmed histologically. Massive metastasis was observed in mice of the control group and the MMC group, but not in the 30 mg/kg TNP-470 group (Fig. 3). No histological evidence of a therapeutic effect of MMC or TNP-470, such as tumor-cell necrosis, was observed.

Toxicity In the mice treated with 15 mg/kg and 30 mg/kg TNP-470, a decrease of weight gain was observed, whereas this was not observed in the MMC group (data not shown). The histology of the liver, the kidney and the lung in nude mice administered 30 mg/kg TNP-470 showed no apparent degenerative effects of TNP-470. No death due to drug-induced toxicity was observed in any group.

DISCUSSION

Since solid tumor growth is reported generally to depend on angiogenesis,[13] angiogenesis inhibitors should have an inhibitory effect on *in vivo* tumor growth. The angiogenesis inhibitor TNP-470 has been reported to have inhibitory activities against both tumor growth and metastasis.[8, 14] It was recently shown that TNP-470 inhibited lung and liver metastasis in human choriocarcinoma[5] and colon cancer.[12, 15] However, its inhibitory effect has not previously been examined in liver-metastasizing gastric carcinoma lines in nude mice.

In the present study, we investigated the inhibitory effect of TNP-470 on the tumor growth and liver metastasis of human gastric carcinoma, using our established model, AZ-H5c. Since AZ-H5c was given by intrasplenic injection, this model does not represent the entire scope of hematogenous liver metastasis of gastric carcinomas. But AZ-H5c may reflect the angiogenesis phase of tumor growth at the metastatic site. As anti-metastatic therapy, anti-angiogenic agents are more likely to be useful than chemotherapeutic agents, because neovascularization is essential for tumor micro-metastasis to become established and grow. Our results suggested that liver metastasis could be completely prevented by TNP-470. Perhaps the growth of AZ-H5c tumor cells arriving in the liver after intrasplenic injection was inhibited by the anti-angiogenic action of TNP-470. Almost all of the control group showed metastasis to mesenteric lymph nodes, but none of the 30 mg/kg TNP-470 group showed metastasis to mesenteric lymph nodes (data not shown), indicating that TNP-470 might inhibit lymph node metastasis, as well as liver metastasis.

In this study, TNP-470 appeared to have no effect on tumor growth. Konno *et al.*[12] reported the same result, and suggested that this is consistent with the hypothesis that a rapidly proliferating tumor is more angiogenesis-dependent.[16]

It is well known that MMC shows a strong cytotoxic effect on tumor cells both *in vitro* and *in vivo* by inducing G2 arrest. MMC was selected as the chemotherapeutic agent for the present study because it is frequently used to treat gastric carcinoma, and because Iigo *et al.*[17] reported that it was more effective against liver metastasis induced by the intrasplenic injection of colon 26 cells than against s.c. tumors. In this present study, the anti-metastatic effect of MMC was not as strong as that of TNP-470. However, MMC inhibited the splenic tumor growth to about 1/5 of that observed in the 30 mg/kg TNP-470 group. For clinical application of TNP-470, it may be necessary to use a combination therapy of TNP-470 plus chemotherapeutic agents after radical operation.[18, 19]

Finally, a decrease of body weight was observed in the TNP-470 groups in this study. Yanase *et al.*[5] did not observe weight loss, and Yamaoka *et al.*[8, 14] reported that the effect of TNP-470 on body weight seemed to depend on the type of tumor. Weight loss was the only side effect of TNP-470 noted in this study, and no significant pathohistological findings were observed in various organs of mice.

In conclusion, the angiogenesis inhibitor TNP-470 has a strong inhibitory activity against *in vivo* liver metastasis of human gastric carcinoma, but had no effect on the tumor growth. TNP-470 seems to be a potent anti-metastatic agent for gastric carcinoma, and it may be clinically applicable after further study.

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