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Inhibition of Liver Metastasis of Human Pancreatic Carcinoma by Angiogenesis Inhibitor TNP-470 in Combination with Cisplatin

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The anti-tumor and anti-metastatic effects of O-(chloroacetyl-carbamoyl) fumagillol (TNP-470), an angiogenesis inhibitor, and cisplatin (CDDP), an anti-neoplastic agent, were investigated using our established liver-metastasizing pancreatic carcinoma line, HPC-3H4. HPC-3H4 was injected into the spleens of nude mice. Mice were randomly divided into 5 groups; a control group given saline solution, a group receiving 45 mg/kg TNP-470, a group receiving 90 mg/kg TNP-470, a group receiving 90 mg/kg TNP-470 in combination with 0.25 mg/kg CDDP, and a group receiving 0.25 mg/kg CDDP. In the control group, liver metastasis developed in 14 of 15 mice (93.3%). Liver metastasis developed in 9 of 11 mice (81.8%) receiving 0.25 mg/kg CDDP. It developed in 11 of 15 mice (73.3%) receiving 45 mg/kg TNP-470, in 17 of 18 mice (94.4%) receiving 90 mg/kg TNP-470, and in 4 of 10 mice (40%) receiving 90 mg/kg TNP-470 in combination with 0.25 mg/kg CDDP. TNP-470 in combination with CDDP displayed a significant inhibitory effect on liver metastasis compared to the control. Although TNP-470 alone and CDDP alone had no effect on the tumor growth *in vivo*, 90 mg/kg TNP-470 in combination with 0.25 mg/kg CDDP had a significant effect. *In vitro* examinations demonstrated that the growth of HPC-3H4 cells was only mildly inhibited by TNP-470, but the production of vascular endothelial growth factor (VEGF) by HPC-3H4 was clearly inhibited by TNP-470. The inhibitory effect on the production of VEGF was not strong with CDDP treatment. These results indicate that the angiogenesis inhibitor TNP-470 in combination with low-dose CDDP has inhibitory activity against liver metastasis of human pancreatic carcinoma.

Key words: Metastasis — Human pancreatic carcinoma — TNP-470 — Angiogenesis — Cisplatin

Liver metastasis is very often observed in human pancreatic carcinoma, which is the fifth leading cause of cancer death in Japan and the United States.[1–3] To improve the 5-year survival rate of pancreatic carcinoma, more extensive surgery and combination chemotherapy[4–6] should be attempted to prevent lymph node metastasis and liver metastasis. In addition, there is a need for a new therapy to inhibit tumor-specific angiogenesis. The establishment of relevant animal metastatic models of pancreatic carcinoma is highly important in the search for the development of such new therapeutics for pancreatic carcinoma. We established cell lines, designated HPC-3H1, HPC-3H2, HPC-3H3 and HPC-3H4, with various metastatic potentials in the nude mouse liver by using intrasplenic injections of human pancreatic carcinoma line HPC-3 (manuscript in preparation).

The growth of solid tumors is generally dependent on angiogenesis.[7] This suggests that the process of micrometastasis, especially the growth of metastatic cells, is also dependent on angiogenesis at the metastatic site and that inhibition of angiogenesis may become an effective therapy for metastasis of solid tumors.

O-(Chloroacetyl-carbamoyl) fumagillol (TNP-470), an analog of fumagillin derived from *Aspergillus fumigatus*, inhibits angiogenesis both *in vivo* and *in vitro*, regardless of the presence of angiogenesis factors. It is also less toxic than fumagillin.[8] In addition, it has been reported that TNP-470 has an inhibitory effect on the growth and metastasis of human cell lines[9–12] and rodent tumors.[13] In this study, we investigated the effects of TNP-470 and cisplatin (CDDP), a chemotherapeutic agent, against human pancreatic carcinoma cell line HPC-3H4, which had the highest metastatic potential in the liver.

MATERIALS AND METHODS

Drugs TNP-470 was a kind gift of Takeda Chemical Industries, Ltd. (Osaka). Its structure, disposition and metabolism have already been reported.[14, 15] In *in vivo* experiments, TNP-470 was suspended in a vehicle composed of 0.5% ethanol plus 5% gum arabic in saline. In *in vitro* experiments, TNP-470 was dissolved in dimethylsulfoxide and RPMI-1640 (GIBCO, Grand Island, NY). CDDP (Nippon Kayaku, Tokyo) was dissolved in saline.

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

Cell lines A human pancreatic carcinoma line with high metastatic potential in the liver, designated HPC-3H4, was established by *in vivo* stepwise selection according to the method developed by Morikawa *et al.*[16] in our laboratory. Briefly, 2×10^6 cells of a human pancreatic carcinoma line, HPC-3, were injected into the spleen of nude mice. Cells from liver metastatic foci of injected mice were expanded *in vitro* and subsequently again injected into the spleen of nude mice. By repeating these procedures four times, we obtained a cell line, HPC-3H4, with a high liver metastatic capability. Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords by means of 0.25% trypsin digestion (DIA-IATRON, Tokyo). Murine endothelial cell line D10 was established *in vitro* from vascular tumors of the liver; the biological features were previously reported.[17] Cells were maintained in RPMI-1640 with 10% fetal calf serum (FCS) (Sigma, St. Louis, MO).

Assay of tumor growth *in vivo* HPC-3H4 cells (2×10^6/0.1 ml in phosphate-buffered saline) were s.c.-injected into nude mice. Mice were divided into 5 groups; a control group given saline solution (n=6), a group receiving 45 mg/kg TNP-470 (n=6), a group receiving 90 mg/kg TNP-470 (n=6), a group receiving 90 mg/kg TNP-470 in combination with 0.25 mg/kg CDDP (n=6), and a group receiving 0.25 mg/kg CDDP (n=9). To administer the drugs, 0.1 ml of TNP-470 was given s.c. on alternate days for 4 weeks from day 1 after s.c. injection, and 0.1 ml of CDDP (0.25 mg/kg) was given i.p. 5 days a week from day 1 after s.c. injection. The resulting tumors were measured with calipers and tumor volume was estimated using the following formula: $V = a\times b^2/2$ (V, volume; a, the longest diameter; b, the shortest diameter).

Assay of liver metastasis Briefly, HPC-3H4 cells (2×10^6/0.1 ml in phosphate-buffered saline) were injected into the spleen of nude mice using a 26-gauge needle. Mice were divided into 5 groups; a control group given saline solution (n=15), a group receiving 45 mg/kg TNP-470 (n=15), a group receiving 90 mg/kg TNP-470 (n=18), a group receiving 90 mg/kg TNP-470 in combination with 0.25 mg/kg CDDP (n=10), and a group receiving 0.25 mg/kg CDDP (n=11). The TNP-470 (45 or 90 mg/kg) was given s.c. on alternate days for 4 weeks from day 1 after intrasplenic injection and 0.1 ml of CDDP (0.25 mg/kg) was administered i.p. 5 days a week from day 1 after the intrasplenic injection. Mice were killed approximately 4 weeks after intrasplenic injection. The numbers of liver metastases and metastatic liver tumors were estimated histologically and macroscopically.

Cell proliferation assay *in vitro* HPC-3H4, D10 and HUVEC cells (1×10^4/well) were suspended in RPMI-1640 containing 10% FCS, and cultured in a 96-well plate. Culture supernatants were replaced the next day (day 1) with fresh medium, containing TNP-470 or CDDP at concentrations of 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^2, 10^3 and 10^4 ng/ml. On day 4, to count viable cells, an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] assay was performed. The MTT assay was previously described.[18] Briefly, 10 μl of 5 mg/ml MTT was added and the cells were incubated for 4 h. A purple formazan product was then formed by the action of mitochondrial enzymes in living cells. This product was solubilized by the addition of acidic isopropanol. The absorbance of each well was quantified with a NovaPath microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. Growth rate was calculated for each concentration as (absorption of the experimental well on day 4/absorption of the control on day 4).

Vascular endothelial growth factor (VEGF) production HPC-3H4 cells were cultured in 96-well plates at 1×10^4 cells/well in RPMI-1640 medium with 10% FCS. Culture media were changed to RPMI-1640 containing no FCS but various concentrations of TNP-470 or CDDP. After 2 days, supernatants were collected and concentrations of VEGF were measured by using VEGF enzyme-linked immunosorbent assay kits (IBL, Fujioka).

Statistical analysis The χ^2 test was used to compare the number of mice with liver metastasis in each group. The unpaired t test was used for other experiments. A P-value of less than 0.05 was considered statistically significant.

RESULTS

Subcutaneous tumor growth After inoculation of HPC-3H4 cells, the subcutaneous tumor growth was evaluated to determine the inhibitory effect of TNP-470. Fig. 1 shows the subcutaneous tumor volume in the control group, the TNP-470 group, the TNP-470 in combination with CDDP group, and the CDDP group. Although TNP-470 alone and CDDP alone did not inhibit the subcutaneous tumor growth, TNP-470 in combination with CDDP significantly inhibited it (P<0.01).

Liver metastasis Fig. 2 illustrates the effects of TNP-470 and CDDP on liver metastasis. The liver metastatic rate was 93.3% in the control group. No inhibitory effect on liver metastasis was observed in the TNP-470 alone group or CDDP alone group. Liver metastasis developed in 4 of 10 mice (40%) receiving 90 mg/kg TNP-470 in combination with 0.25 mg/kg CDDP, and TNP-470 in combination with CDDP displayed a significant inhibitory effect on liver metastasis compared to the control (P<0.05).

Table I demonstrates the effects of TNP-470 and CDDP on the number of liver metastatic foci. In the group that

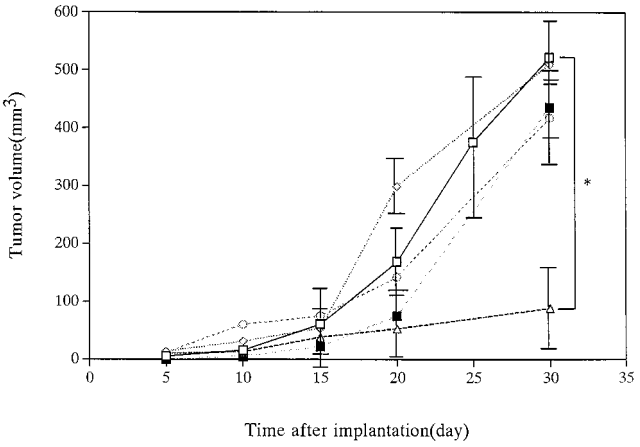

Fig. 1. Subcutaneous tumor growth volume. The combination of 90 mg/kg TNP-470 with 0.25 mg/kg CDDP significantly inhibited tumor growth as compared with the control (P=0.00627). Bars represent mean±standard error. Control (□), 45 mg/kg TNP-470 (◇), 90 mg/kg TNP-470 (○), 90 mg/kg TNP-470+0.25 mg/kg CDDP (△), 0.25 mg/kg CDDP (■).

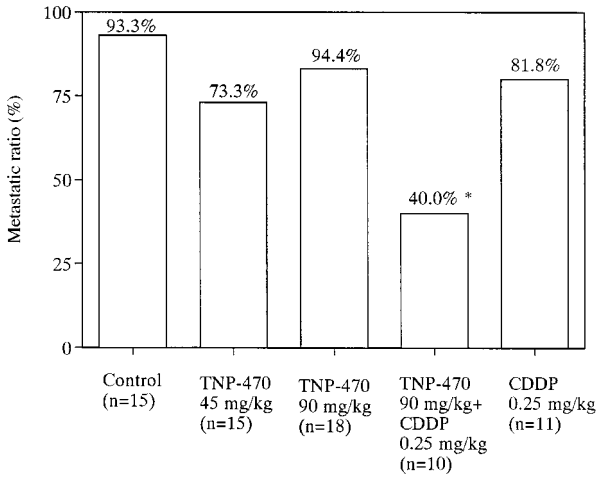

Fig. 2. Inhibitory effect of TNP-470 on liver metastasis. TNP-470 in combination with CDDP significantly inhibited liver metastasis as compared with the control. * Significantly different from the control group (P=0.014).

had more than 30 liver metastatic foci, 90 mg/kg TNP-470 and 90 mg/kg TNP-470 in combination with 0.25 mg/kg CDDP significantly inhibited the number of metastatic foci compared to the control (P<0.001).

All the metastatic lesions were confirmed histologically. Massive metastasis was observed in most mice of the control group, as well as in the TNP-470-alone and CDDP-alone groups, but not in some mice of the TNP-470 in combination with CDDP group (Fig. 3). No distinct histological evidence of a therapeutic effect of TNP-470 and CDDP, such as tumor-cell necrosis, was observed.

Table I. Number of Metastatic Liver

Group	Total number of mice	0	0<foci<30	30≤foci
Control	15	1/15	0/15	14/15
TNP-470 45 mg/kg	15	4/15	0/15	11/15
TNP-470 90 mg/kg	18	3/18	12/18	3/18*
TNP-470 90 mg/kg +CDDP 0.25 mg/kg	10	6/10	2/10	2/10*
CDDP 0.25 mg/kg	11	2/11	0/11	9/11

The effect of TNP-470 against number of metastatic liver foci. 90 mg/kg TNP-470 and 90 mg/kg TNP-470 in combination with 0.25 mg/kg CDDP inhibited significantly the number of liver metastatic foci as compared with the control. * Statistically significant difference from group (P<0.001).

Toxicity There was no obvious weight loss with statistical significance during the treatment period in any group (Fig. 4). The histology of the liver, the kidney, the lung and the heart in nude mice administered TNP-470 appeared to show no degenerative effects of TNP-470. No deaths due to drug-induced side effects were observed in any group.

Cell proliferation assay *in vitro* To assess the effect of TNP-470 on the growth of vascular cells and tumor cells, we utilized HUVEC umbilical cells, D10 mouse vascular endothelial tumor cells, and HPC-3H4. As shown in Fig. 5, the growth of HUVEC cultured with TNP-470 was inhibited in an almost dose-dependent manner. It appeared that the D10 growth was also inhibited to some extent. Similarly, as demonstrated in Fig. 6, the growth of HPC-3H4 pancreatic metastatic tumor cells was apparently reduced with TNP-470, although the effect was not large. Meanwhile, it appeared that the growth of HPC-3H4 was clearly inhibited by CDDP at higher concentrations such as 10^3 to 10^4 ng/ml.

VEGF production We also determined the VEGF concentration produced by HPC-3H4 cells upon treatment with TNP-470 or CDDP. As shown in Fig. 7, it was significantly inhibited by TNP-470 as compared with the control (P<0.01) at concentrations ranging from 10^{-4} to 10^3 ng/ml. On the other hand, the production was inhibited less effectively by CDDP than by TNP-470. It seemed that CDDP also reduced the VEGF production by HPC-3H4 at concentrations of 10^{-2} to 10^3 ng/ml. However, this reduction probably resulted from the growth inhibition of HPC-3H4 by CDDP.

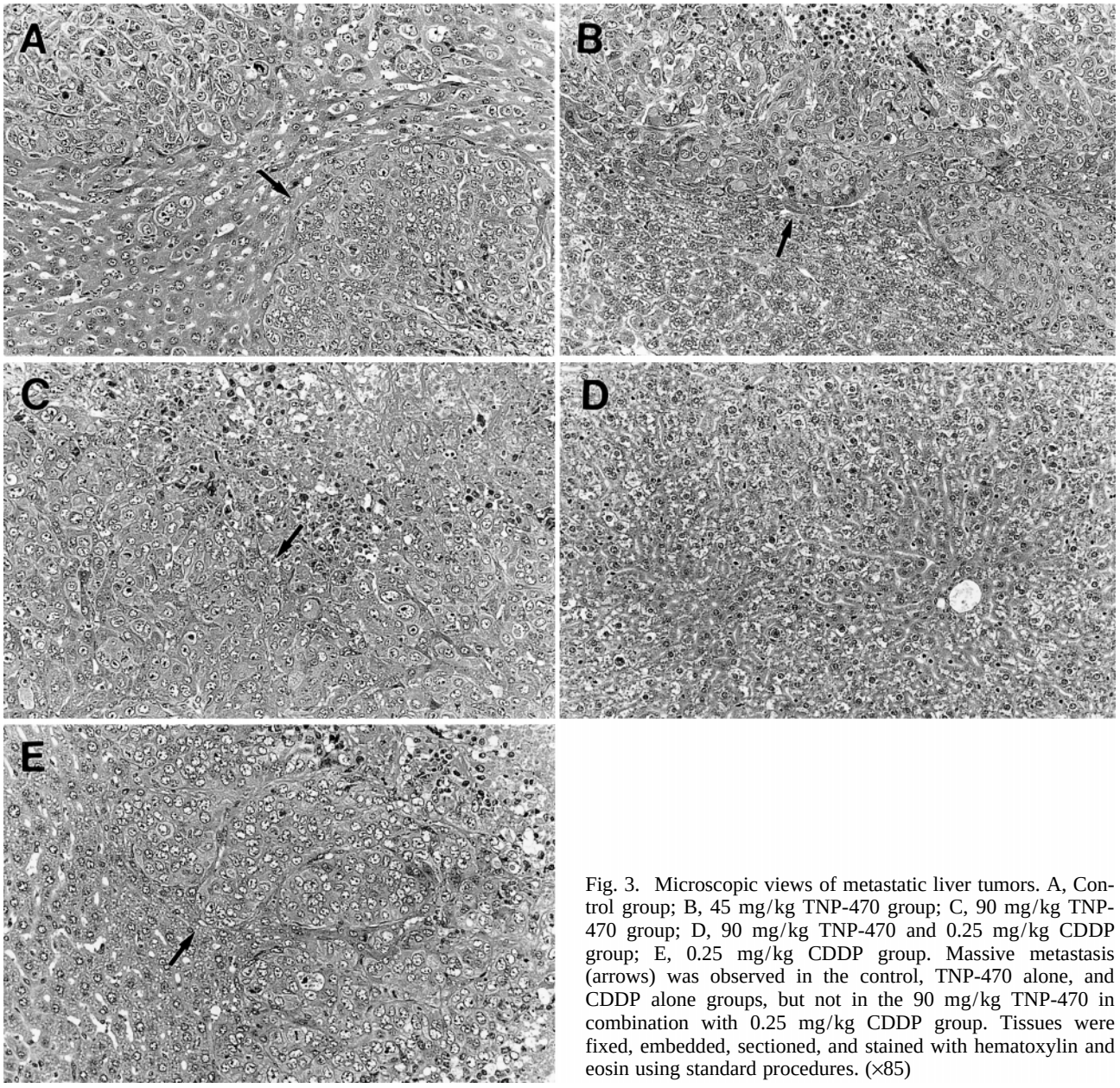

Fig. 3. Microscopic views of metastatic liver tumors. A, Control group; B, 45 mg/kg TNP-470 group; C, 90 mg/kg TNP-470 group; D, 90 mg/kg TNP-470 and 0.25 mg/kg CDDP group; E, 0.25 mg/kg CDDP group. Massive metastasis (arrows) was observed in the control, TNP-470 alone, and CDDP alone groups, but not in the 90 mg/kg TNP-470 in combination with 0.25 mg/kg CDDP group. Tissues were fixed, embedded, sectioned, and stained with hematoxylin and eosin using standard procedures. (×85)

DISCUSSION

The angiogenesis inhibitor TNP-470 has been reported to inhibit neovascularization by preventing endothelial cell proliferation.[13, 19] One possible mechanism for its effect is that it acts on endothelial cells to inhibit growth factor-induced DNA synthesis. It exhibits potent inhibition of endothelial cell growth, with half-maximum inhibition occurring at approximately 10 pg/ml.[14] TNP-470 has inhibitory activities against both tumor growth and metastasis.[13, 20] Antitumor and antimetastatic activities of TNP-470 were evaluated in various human cell lines,[21] and we also reported the inhibitory effect of liver metastasis by using a human gastric carcinoma cell line, AZ-H5c.[12] To our knowledge, this is the first report that describes the anti-metastatic activity of TNP-470 in combination with CDDP in a human pancreatic carcinoma line.

In this study, we investigated the inhibition of tumor growth and liver metastasis of human pancreatic carcinoma using our established model, HPC-3H4. This cell

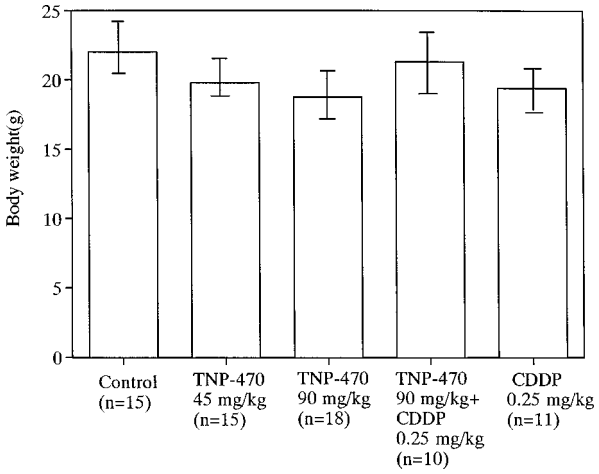

Fig. 4. Body weights of nude mice 4 weeks after splenic injection. The weight gain of each group was not decreased compared with the control. Bars represent mean±standard error.

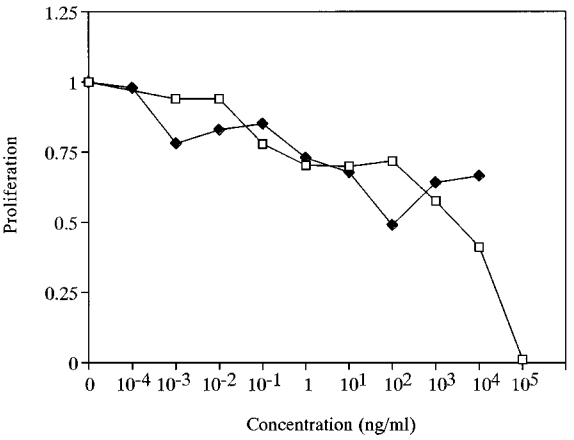

Fig. 6. Effects of TNP-470 (◆) and CDDP (□) on *in vitro* proliferation of HPC-3H4. The proliferation is shown as the ratio of the growth for each concentration with/without TNP-470.

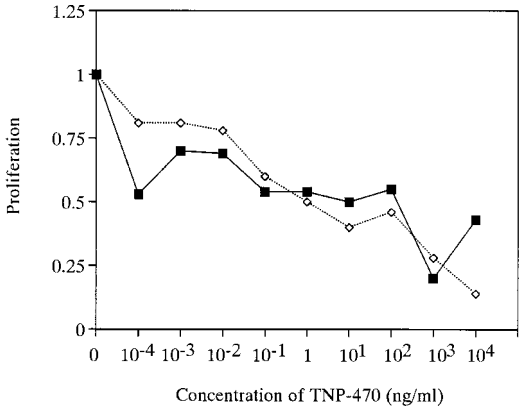

Fig. 5. Effects of TNP-470 on *in vitro* proliferation of D10 (■) and HUVEC (◇). The proliferation is shown as the ratio of the growth for each concentration with/without TNP-470.

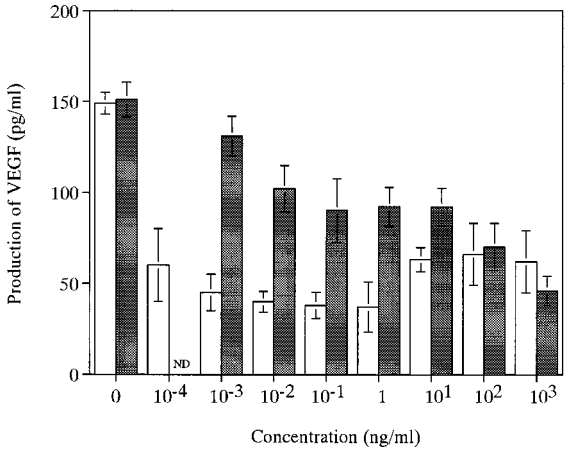

Fig. 7. Effects of TNP-470 and CDDP on production of VEGF by HPC-3H4. The open and shaded columns denote TNP-470 and CDDP, respectively. Bars represent mean±standard error. ND: not determined.

line does not represent the entire scope of hematogenous liver metastasis of pancreatic carcinomas, but HPC-3H4 may reflect the angiogenesis phase of tumor growth at the metastatic site. Generally, it has been shown that pancreatic carcinoma forms hypovascular tumors with fibrosis and strong invasiveness. However, TNP-470 was used in the light of the idea that tumor growth and metastasis require the development of new vessels.[22, 23] It is rapidly cleared from the circulation with a short terminal half-life (0.88±2.5 h).[24] Thus, for treatment of pancreatic carcinoma, which, because of its high biological malignancy, requires prolonged maintenance of effective blood concentrations of drugs, we should employ combination chemotherapy.

The combined effects of TNP-470 and various chemotherapeutic agents have recently been investigated. Kato *et al.*[19] reported significant combinative effects of TNP-470 with mitomycin-C, adriamycin, CDDP, and 5-fluorouracil (5-FU) in mouse models. Yamaoka *et al.*[20] showed that the effect of TNP-470 against prostate carcinomas

was enhanced by combination with CDDP. These results suggest that TNP-470 is likely to be effective in combination with a chemotherapeutic agent, and combination therapy is very important for treatment of pancreatic carcinoma. Because CDDP is frequently used to treat pancreatic carcinoma, it was selected as the chemotherapeutic agent for the present study. It is well known that CDDP inhibits DNA synthesis and the division of tumor cells, but high-dose CDDP has severe side effects. Recently, daily low-dose CDDP has been used clinically against solid tumors.[25, 26] In the present study, daily low-dose (0.25 mg/kg) CDDP *in vivo* was administered i.p. 5 days a week; at this concentration, CDDP showed neither a reduction of the subcutaneous HPC-3H4 tumor growth nor side effects. The anti-metastatic and anti-tumor effects of the combination of TNP-470 and CDDP were stronger than those of TNP-470 alone or CDDP alone. Although the mechanism of this combination therapy is not clear, it appeared to be due to a synergistic effect.

The growth of HPC-3H4 cells *in vitro* was only mildly inhibited by TNP-470, even with high concentrations such as 10^3 and 10^4 ng/ml. This is consistent with the fact that the tumor growth of HPC-3H4 was not inhibited by 90 mg/kg TNP-470. Meanwhile, the production of VEGF by HPC-3H4 cells was significantly inhibited by TNP-470 rather than CDDP.

Although TNP-470 inhibited the production of VEGF by HPC-3H4 cells *in vitro*, 90 mg/kg TNP-470 alone did not inhibit liver metastasis *in vivo*. We previously reported that 30 mg/kg TNP-470 completely inhibited liver metastasis of the human gastric carcinoma line AZ-H5c.[12] This difference may have been due to the fact that HPC-3H4 has higher biological malignancy than AZ-H5c with regard to the growth activity, the liver metastatic rate, the adhesive activity to extracellular matrix and the production of VEGF (data not shown).

Finally, no weight loss was observed in the TNP-470 groups and the combination group with TNP-470 and CDDP as compared to the control in this study. Yamaoka *et al.*[13, 20] reported that the effect of TNP-470 on body weight seemed to depend on the type of tumor. Weight loss was reported as the main side effect of TNP-470, but there were no significant pathohistological injuries in various organs of mice. Consequently, the low toxicity may be convenient for application of combination therapy employing TNP-470 with a chemotherapeutic agent.

In conclusion, the combination of the angiogenesis inhibitor TNP-470 and low-dose CDDP had an inhibitory effect against liver metastasis of a human pancreatic carcinoma line *in vivo*. TNP-470 in combination with daily low-dose CDDP seems to be a promising candidate for development as an anti-metastatic agent for pancreatic carcinoma, and it may be clinically applicable after further study. In the future, to elucidate precisely the clinical implications of treatment with TNP-470, we should further investigate the period of medication, the dose and various combinations. It may be necessary to employ a combination therapy using TNP-470 plus a chemotherapeutic agent after resection.[27, 28]

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