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Regression of Metastatic Liver Tumors in Rats Treated with Angiogenesis Inhibitor TNP-470: Occurrence of Apoptosis and Necrosis

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To clarify the mechanism of the reduction of metastatic liver tumors in rats treated with angiogenesis inhibitor TNP-470, the death of tumor cells was examined pathologically and ultrastructurally. Liver metastases were developed by intravenous injection of AH-130 cells. TNP-470 was given subcutaneously after tumor cell injection. Alterations in the size and number of metastatic tumors were examined at various time points, in association with the analysis of cell death pattern. The metastatic nodules were divided into 4 groups according to the morphological patterns of cell death; no cell death, scattered apoptosis, central necrosis, and diffuse necrosis. The number and size of the metastatic tumors at 2 weeks in untreated rats were larger than those in treated rats. The number of tumors in untreated rats decreased, but the tumor size increased. All rats treated with TNP-470 were alive and free from tumors after 4 weeks, whereas all the untreated rats died of liver metastases. The percentages of the tumors with necrosis in untreated rats (61.2% at 2 weeks and 100% at 4 weeks) were significantly higher than that (31.8% at 2 weeks) in treated rats ($P < 0.01$). The percentage of the tumors containing apoptotic cells in treated rats was significantly higher than that in untreated rats (54.5% vs. 30.6%; $P < 0.05$). The growth of metastatic tumors without treatment might be faster than the growth of vessels in untreated tumors, resulting in central necrosis due to ischemia. On the other hand, the reduction of metastatic liver tumors treated with TNP-470 might be caused by inhibition of angiogenesis, providing a weak ischemic stimulus which triggers apoptosis, rather than by a direct cytotoxic effect on tumor cells, because previous *in vivo* experiments demonstrated that TNP-470 affected endothelial cells but not tumor cells.

Key words: Apoptosis — Necrosis — TNP-470 — Angiogenesis — Metastasis

Antiangiogenic therapy is effective against tumor growth and metastasis.[1–3] TNP-470, a synthetic analog of fumagillin, isolated from *Aspergillus fumagatus*, is an active antiangiogenic agents,[4] but although *in vitro* and *in vivo* experimental evidence shows that it is effective against metastatic liver tumor, its mechanism of action is still obscure. Since TNP-470 is a potent inhibitor of endothelial proliferation,[5] it has been proposed that an ischemic stimulus prevents tumor growth. Our previous study demonstrated that metastatic liver tumors regressed even after treatment with TNP-470.[2,3] This suggests that the administration of TNP-470 may induce crucial damage to tumor cells, though TNP-470 is not cytotoxic.[1]

Tumor growth is regulated by the balance between cell proliferation and cell death.[6,7] Both necrosis and apoptosis influence tumor regression, and can be induced by ischemia, chemotherapeutic agents and irradiation. A weak ischemic stimulus is well known to trigger apoptosis.[8] Since it was reported that apoptotic bodies were far less common in the liver of AH-130-bearing rats during the hyperplastic phase compared with the regressive phase,[9] we hypothesized that disturbance of neovascularization by TNP-470 might enhance apoptotic cell death. In the present study, we treated an *in vivo* animal model with TNP-470, and histopathologically examined the pattern of cell death in metastatic liver tumors to clarify the mechanism of tumor regression.

MATERIALS AND METHODS

Reagents TNP-470 (AGM-1470), a synthetic analog of fumagillin, was a generous gift from Takeda Chemical Industries (Osaka). It was suspended in a vehicle of 1% ethanol plus 5% gum arabic in saline. Serum-free RPMI 1640 medium (Nissui Seiyaku Co., Tokyo) was used for cell culture.

Animals Male Donryu rats, weighing 100–120 g, were purchased from SLC, Inc. (Hamamatsu). Rats were housed in stainless steel cages under temperature- and humidity-controlled conditions with a 12 h light-dark cycle. They were maintained on a standard rat pellet diet with tap water *ad libitum*. The experimental protocol was conducted in accordance with the Manual of

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Hamamatsu University School of Medicine. Four or more animals were used at each experimental time point.

Tumors The AH-130 cell line was a gift from Kyowa Hakko Ltd. (Fuji) and was maintained by serial intraperitoneal implantation in male Donryu rats. The hepatic metastasis model was established according to the method described previously.[3]

Experimental design In this experiment 50 rats were used, 25 in the untreated group and 25 in the treated group. The animals of the untreated group received the vehicle only (1% ethanol plus 5% gum arabic in saline). The animals of the treated group received TNP-470 at a dose of 15 mg/kg body weight subcutaneously every other day (7 times); the first dose (injection volume, 0.6 ml) was given 24 h after AH-130 implantation, as described previously.[2, 3] On the 14th day, 14 rats (untreated 7, treated 7) were killed and the whole livers were immediately removed and processed for further studies. Remaining rats were killed at intervals of 2 weeks for 2 months.

Preparation of samples The whole liver and paraaortic lymph node were excised, weighed and immediately fixed in 10% buffered formalin for hematoxylin and eosin (HE) staining. For electron microscopic study, the liver was cut into small pieces, which were fixed in 2% glutaraldehyde fixative for about 2–4 h, washed with phosphate-buffered saline (PBS), and kept in PBS solution for a period of 2–4 h.

Histological and ultrastructural studies Fixed samples were dehydrated in graded ethanol solutions before paraffin embedding. Sections were cut at 3 μm for HE staining. For electron microscopic examination, glutaraldehyde-fixed samples were postfixed in 2% osmium tetroxide and embedded in Epon. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate. Ultrastructural observations were performed with a Hitachi H-600 electron microscope.

Classification of cell death patterns Based on morphological criteria, cell death was defined as apoptosis or necrosis. The distinctive morphological features of apoptosis, as described by Kerr *et al.*[10, 11] and Walker *et al.*,[12] were used to recognize apoptotic cells. Necrotic cell death was identified on the basis of the descriptions of Kerr *et al.*[13] and Wyllie *et al.*[14]

According to the extent and pattern of cell death, metastatic tumors were divided into 4 groups; no cell death, scattered apoptosis, central necrosis, and diffuse necrosis.

Analysis of the mean diameter and cell death pattern The relationship between the mean diameter of the metastatic tumors and the pattern of cell death was examined.

Statistical analysis Data were analyzed by the use of Student's t test and the χ^2 test with the criterion of significance set at $P<0.05$.

RESULTS

Alteration of the number of metastatic nodules in the liver The number of metastatic liver nodules per rat peaked at 2 weeks after injection of AH-130 cells in both treated and untreated rats (Table I). At this time, the mean number of metastatic nodules in untreated rats was significantly higher than that in TNP-470-treated rats ($P<0.01$). The mean numbers of metastatic nodules per rat were 19.1 in untreated rats and 3.1 in rats treated with TNP-470. The number of nodules in the untreated rats declined to low levels at 4 weeks after cell injection, whereas there were no metastatic nodules in the treated rats after 4 weeks. All untreated rats died of liver metastases after 6 weeks, whereas all rats treated with TNP-470 were free from tumors.

Mean diameter of metastatic tumors The diameters of the metastatic tumor in the liver at 2 weeks were 1176$\pm$608 μm ($n=134$) in untreated rats and 689$\pm$307 μm ($n=22$) in treated rats. The metastatic nodules grew gradually (1883$\pm$843 μm, $n=53$) in the untreated rats although their number was decreased at 4 weeks.

Histopathological and ultrastructural findings Histological examination of the metastatic nodules revealed two patterns of cell death in the tumors; apoptosis and necrosis. Apoptotic cells were present randomly and singly among the tumor cells (Fig. 1). The ultrastructural examination of apoptotic cells showed disintegration, membrane blebbing, and contraction in size. The nuclei of the affected cells exhibited dense and amorphous chromatin, and their cytoplasm contained several rough endoplasmic reticulum and mitochondria without Weibel-Palade bodies, indicating that they were derived from AH-130 tumor cells. On the other hand, necrosis was present in the center of the tumors (Fig. 2).

Relationship between the mean diameter of metastatic tumors and the pattern of cell death The data are summarized in Fig. 3. The mean diameters of tumors with necrosis were greater than those of tumors with apoptosis or without cell death. The nodules, measuring

Table I. The Number of Metastatic Nodules in the Liver of TNP-470-treated and -untreated Rats

Group	2 weeks	4 weeks	6 weeks	8 weeks
Untreated	19.1 ($n=7$)	10.6 ($n=5$)	a)	a)
TNP-470-treated	3.1 ($n=7$)	0 ($n=4$)	0 ($n=5$)	0 ($n=7$)

Values represent the average number of metastatic nodules per rat.

a) All experimental rats died of metastatic tumor or associated diseases.

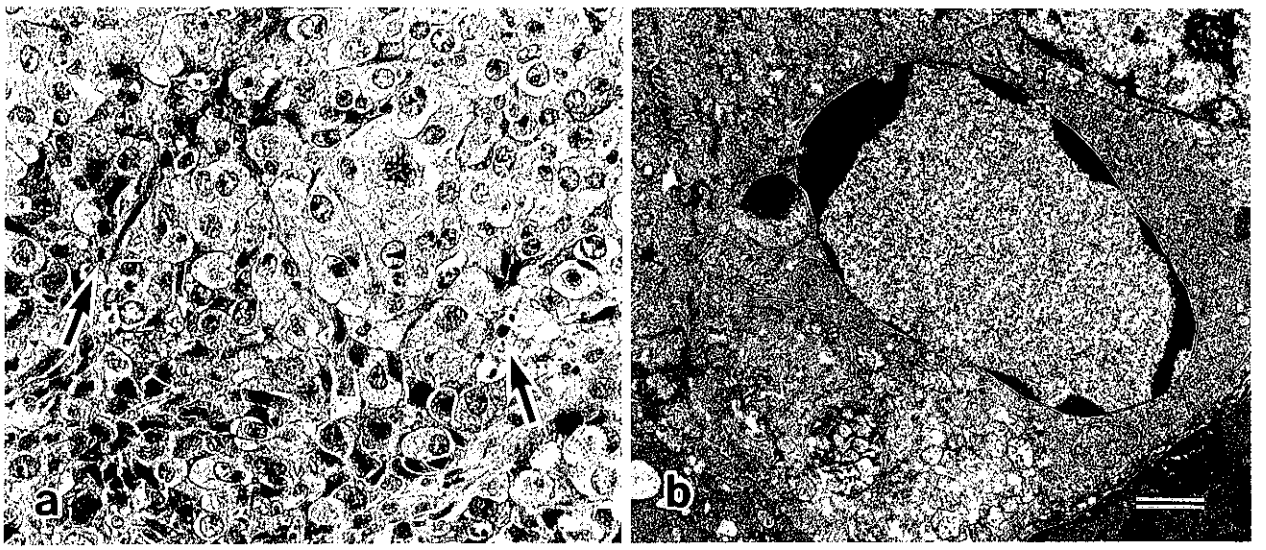

Fig. 1. Microscopic and ultrastructural photographs of metastatic liver nodule with apoptosis in a rat treated with TNP-470 at 2 weeks after implantation. a, Histology of the metastatic nodule with scattered apoptosis. HE ×350. b, Electron microscopic features of AH-130 cell undergoing apoptosis. Note the characteristic nuclear feature of apoptosis. Bar=2 μm.

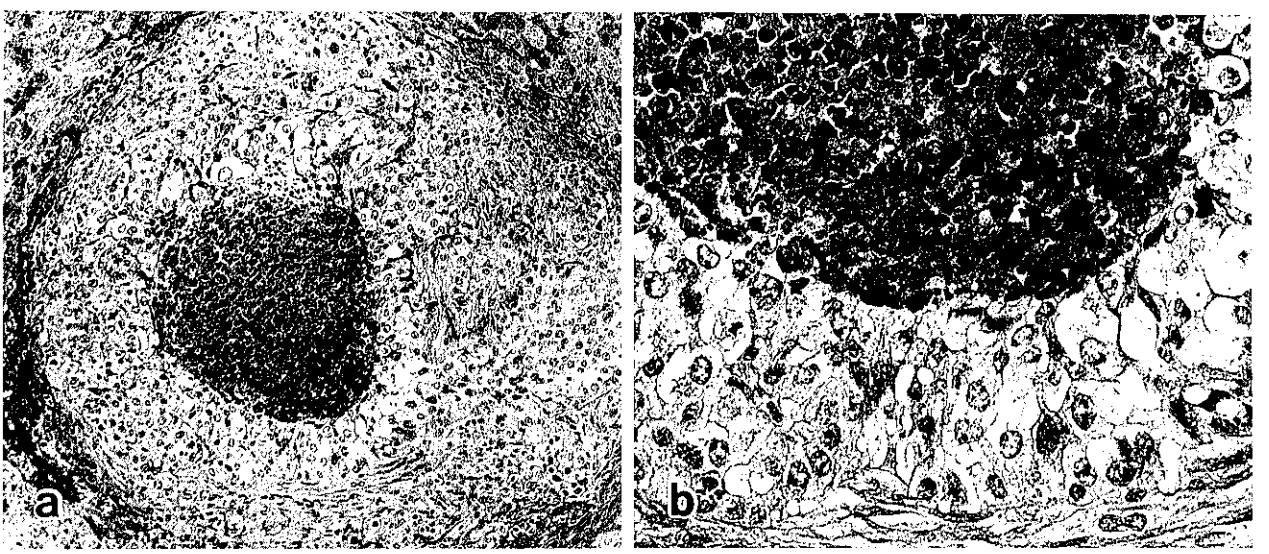

Fig. 2. a, Histology of central necrosis in an untreated rat at 2 weeks after implantation. Massive necrosis is seen in the central area of the tumor. HE ×110. b, High magnification of central necrosis. Necrotic tissue was found in the upper field. HE ×300.

more than 1000 μm in diameter, tended to exhibit central or diffuse necrosis in the untreated rats. There were no tumors showing central or diffuse necrosis in the rats treated with TNP-470. The percentage of combined central and diffuse necrosis at 4 weeks was much higher than that at 2 weeks in the untreated rats (Table II). The total percentages of tumors with necrosis (central or diffuse) in untreated rats were 61.2% at 2 weeks and 100% at 4 weeks, whereas that in treated rats was 31.8% at 2 weeks. On the other hand, the frequencies of scattered apoptosis in untreated and treated rats were 30.6% and 54.5%, respectively. The prevalence of scattered apoptosis in TNP-470-treated rats was significantly higher than that in untreated rats ($P<0.05$).

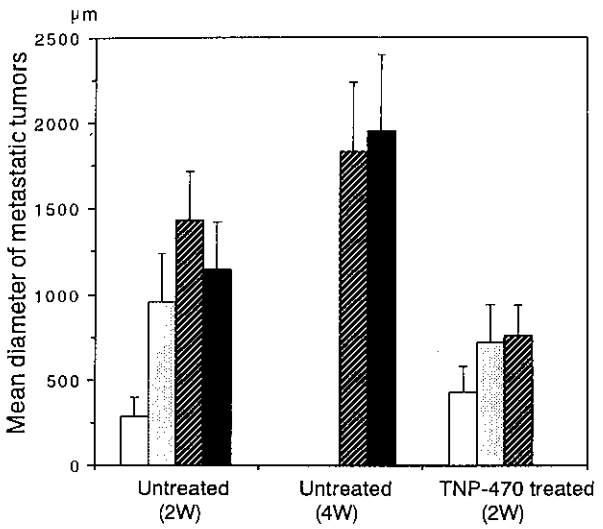

Fig. 3. Relationship between the mean diameter of the metastatic nodules and the patterns of cell death. □, No cell death, ▨, scattered apoptosis, ▨, central necrosis, ■, diffuse necrosis. The data represent means±SD.

Table II. Classification of the Liver Metastatic Tumors according to Cell Death Pattern in TNP-470-treated and -untreated Rats

Group	No cell death	Scattered apoptosis	Central necrosis	Diffuse necrosis
Untreated (2W) (n=134)	11 (8.2%)	41 (30.6%)	76** (56.7%)	6** (4.5%)
Untreated (4W) (n=53)	0	0	30** (56.6%)	23** (43.4%)
TNP-470-treated (2W) (n=22)	3 (13.7%)	12* (54.5%)	7 (31.8%)	0

Values represent the number of metastatic nodules in the liver with the percentage in parentheses.
* There were significantly more tumors containing apoptosis in treated rats than in untreated rats ($P<0.05$)
** There were significantly more tumors with necrosis in untreated rats than in treated rats ($P<0.01$).

DISCUSSION

Various therapeutic modalities are available for unresectable metastatic liver cancer, including numerous systemic chemotherapeutic agents, hepatic artery chemoembolization, radiation therapy and lymphokine activation of killer cells, but none has achieved significant success. The survival period of patients with advanced malignancy is a year or less, confirming the lack of impact of current therapies on survival.

Antiangiogenic therapy against metastatic cancer has recently been developed. TNP-470, a potent angiogenesis inhibitor, is expected to be an effective anticancer drug, based on its ability to stop tumor growth and metastases and its prolongation of survival in experimental animals.[2,3] The powerful effect of TNP-470 and its few side effects *in vivo* have already led to phase I/II clinical trials in human cancer. Thus, TNP-470 is a promising candidate for metastatic cancer therapy. However, the precise mechanism of the antitumor effect *in vivo* of this drug has not yet been elucidated.

Our data clearly demonstrated that metastatic liver tumors were reduced by treatment with TNP-470. It might be expected that TNP-470 has some cytotoxic effect on tumor cells *in vivo*, like other chemotherapeutic agents. However, no significant effect was noted when it was added to a variety of cancer cell lines, even at concentrations a thousand times higher than that found to be active on endothelial cells.[1] Moreover, TNP-470 can specifically inhibit endothelial cell proliferation by preventing the entry of the cell into the G1 phase of the cell cycle.[5] Therefore, it seems probable that there is some relationship between the tumor reduction *in vivo* and inhibition of angiogenesis.

From the histological viewpoint, the percentage of tumors with necrosis in untreated rats was significantly higher than that in treated rats, while the percentage of the tumors containing apoptotic cells in treated rats was significantly higher than that in untreated rats. Although the incidence of cell death seemed to be low in the metastatic tumors containing apoptotic cells, the present study showed that such tumors disappeared. Therefore, apoptosis may play an important role in the regression of metastatic liver tumors.

The mechanism of disappearance of metastatic nodules in TNP-470-treated rats is still obscure. However, it has been reported that angiogenesis inhibitors control metastatic growth by indirectly increasing apoptosis in tumor cells.[15] We suggest that the angiogenesis inhibitor in our study induces a weak ischemic state which triggers apoptosis of tumor cells, because weak ischemia has been shown to trigger cells to undergo apoptosis.[8,13]

However, it has also been shown that TNP-470 stimulates the host immune system *in vivo*, especially B cell proliferation through T lymphocytes.[16] Thus, cytokines or cell-to-cell interactions may influence the reduction of tumors *in vivo* or the occurrence of apoptosis. Further studies are needed.

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REFERENCES

1) Yanase, T., Tamura, M., Fujita, K., Kodama, S. and Tanaka, K. Inhibitory effect of angiogenesis inhibitor TNP-470 on tumor growth and metastasis of human cell lines *in vitro* and *in vivo*. *Cancer Res.*, **53**, 2566–2570 (1993).
2) Konno, H., Tanaka, T., Matsuda, I., Nakai, T., Maruo, Y., Nishino, N., Nakamura, S. and Baba, S. Comparison of the inhibitory effect of the angiogenesis inhibitor, TNP-470, and mitomycin C on the growth and liver metastasis of human colon cancer. *Int. J. Cancer*, **61**, 268–271 (1995).
3) Ahmed, M. H., Konno, H., Nahar, L., Tanaka, T., Naito, Y., Nakamura, S. and Baba, S. The angiogenesis inhibitor TNP-470 (AGM-470) improves long-term survival of rats with liver metastasis. *J. Surg. Res.*, **64**, 35–41 (1996).
4) Ingber, D., Fujita, T., Kishimoto, S., Sudo, K., Kanamaru, T., Brem, H. and Folkman, J. Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. *Nature*, **348**, 555–557 (1990).
5) Antonie, N., Grerimers, C., Roanne, C. D., Kusaka, M., Heinen, E., Simar, L. J. and Castronovo, V. AGM-470, a potent angiogenesis inhibitor, prevents the entry of normal but not transformed endothelial cells into the G1 phase of the cell cycle. *Cancer Res.*, **54**, 2073–2076 (1994).
6) Wyllie, A. H. The biology of cell death in tumours. *Anticancer Res.*, **5**, 131–136 (1985).
7) Arai, T. and Kino, I. Role of apoptosis in modulation of the growth of human colorectal tubular and villous adenomas. *J. Pathol.*, **176**, 37–44 (1995).
8) Thompson, C. B. Apoptosis in the pathogenesis and treatment of disease. *Science*, **267**, 1456–1462 (1995).
9) Tessitore, L., Valente, G., Bonelli, G., Costelli, P. and Baccino, F. M. Regulation of cell turnover in the livers of tumour-bearing rats: occurrence of apoptosis. *Int. J. Cancer*, **44**, 697–700 (1989).
10) Kerr, J. F. R., Winterford, C. M. and Harmon, B. V. Apoptosis. Its significance in cancer and cancer therapy. *Cancer*, **73**, 2013–2026 (1994).
11) Kerr, J. F. R., Gobé, G. C., Winterford, C. M. and Harmon, B. V. Anatomical methods in cell death. *Methods Cell Biol.*, **46**, 1–27 (1995).
12) Walker, N. I., Harmon, B. V., Gobé, G. C. and Kerr, J. F. R. Patterns of cell death. *Methods Achiev. Exp. Pathol.*, **13**, 18–54 (1988).
13) Kerr, J. F. R., Wyllie, A. H. and Currie, A. R. Apoptosis: a basic biologic phenomenon with wide ranging implications in tissue kinetics. *Br. J. Cancer*, **26**, 239–257 (1972).
14) Wyllie, A. H., Kerr, J. F. R. and Currie, A. R. Cell death: the significance of apoptosis. *Int. Rev. Cytol.*, **68**, 251–306 (1980).
15) Holmgren, L., O'Reilly, M. S. and Folkman, J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat. Med.*, **1**, 149–153 (1995).
16) Antoine, N., Daukandt, M., Heinen, E., Simar, L. J. and Castronovo, V. *In vitro* and *in vivo* stimulation of the murine immune system by AGM-1470, a potent angiogenesis inhibitor. *Am. J. Pathol.*, **148**, 393–398 (1996).