

## Antitumor Effect of a Neutralizing Antibody to Vascular Endothelial Growth Factor on Liver Metastasis of Endocrine Neoplasm

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Distant metastasis of gastrointestinal endocrine neoplasm is resistant to currently available treatments. Because hematogenic metastasis is dominant, anti-angiogenic drugs are expected to be a novel therapy for this neoplasm. In the present study, the therapeutic effect of vascular endothelial growth factor neutralizing antibody (VEGFAb) on liver metastasis of an endocrine neoplasm was investigated experimentally. Cecal transplantation into nude mice of small pieces of EN-1, a xenotransplanted human intestinal endocrine neoplasm, resulted in liver metastasis. A treated group ( $n=19$ ) received 100  $\mu\text{g}/\text{mouse}$  of VEGFAB intraperitoneally on alternate days from day 10 after tumor transplantation, and the control group ( $n=19$ ) received saline. Five of the 19 control mice died of tumor progression, of which 2 could not be evaluated. The cecal tumor weighed  $6316 \pm 2333$  mg ( $n=17$ ) in the control group and  $1209 \pm 837$  mg ( $n=19$ ) in the treated group ( $P<0.01$ ) 6 weeks after transplantation. Liver metastasis developed in 16 of 17 control mice and in 2 of 19 treated mice ( $P<0.01$ ). The VEGF level of the whole cecal tumor in the control group was significantly higher than that in the treated group ( $305.1 \pm 174.1$  vs.  $54.7 \pm 41.2$  mg;  $P<0.001$ ). VEGFAB did not cause any body weight loss ( $28.52 \pm 1.63$  in the control vs.  $28.44 \pm 1.71$  g in the treated group). These results indicate that VEGFAB may be a novel therapeutic agent for endocrine neoplasm with distant metastasis.

Key words: Endocrine neoplasm — Liver metastasis — VEGF — VEGFAB — Angiogenesis

Endocrine tumors include various tumors with malignant potential. Among them, carcinoid tumors have a low-grade malignancy when compared with carcinoma.<sup>1)</sup> On the other hand, small cell undifferentiated carcinoma or stem cell carcinomas consisting of primitive cells with endocrine features are immature and highly aggressive tumors.<sup>2)</sup> Endocrine cell carcinoma is an endocrine tumor with high malignant potential, and in principle is more aggressive than carcinoma.

We established a xenotransplanted human intestinal endocrine neoplasm, EN-1, from a duodenal carcinoid tumor. It retains the immunohistochemical and ultrastructural features of the endocrine tumor. A liver metastatic model of EN-1 was produced by cecal transplantation into nude mice.

Currently available therapeutic options for endocrine tumor include surgery, chemotherapy, radiotherapy, and hormonal manipulation. However, the therapeutic effects of those modalities on distant metastasis of the gastrointestinal endocrine tumor are limited.<sup>3)</sup>

Endocrine neoplasms frequently show hematogenic metastasis. This suggests that anti-angiogenic drugs may represent a novel therapy for this neoplasm, because angiogenic factors reportedly play an important role in the

hematogenic metastasis of human malignancies. We have previously reported the therapeutic effect of an angiogenesis inhibitor on the liver metastasis of human gastrointestinal malignancies.<sup>4-6)</sup>

In the present study, the therapeutic effect of vascular endothelial cell growth factor neutralizing monoclonal antibody (VEGFAB) on the liver metastasis of EN-1 was investigated by using a liver metastatic model, with the aim of developing a novel therapy for endocrine neoplasm.

### MATERIALS AND METHODS

**EN-1 (human endocrine neoplasm)** In January 1990, a duodenal lesion was aseptically resected from a 68-year-old Japanese woman who had liver metastases arising from a duodenal atypical carcinoid. The resected specimen was aseptically transplanted into nude mice, and a cell line was established *in vitro* and designated CT-nu-1. The carcinoid tumor was re-established *in vivo* by subcutaneous injection of CT-nu-1 cells into BALB/c nude mice and designated EN-1. EN-1 has been maintained by subcutaneous passage for about 2 years.

**Animals** Male BALB/c nu/nu mice were obtained from

Clea Japan Animals Inc. (Tokyo) and were used at 5–6 weeks of age.

**Immunohistochemical studies** The original human carcinoid tumor and EN-1, resected at 6 weeks after transplantation, were fixed in 4% buffered formaldehyde (pH 7.35), routinely processed, and embedded in paraffin. The sections were stained with hematoxylin-eosin (HE) and Grimelius' impregnation was performed to demonstrate argyrophilia. Immunohistochemical staining was also done to detect neuron-specific enolase (NSE) and Chromogranin A as endocrine cell markers using rabbit polyclonal NSE antiserum (Dako, Santa Barbara, CA) and polyclonal Chromogranin A antiserum (Dako), respectively. To detect neuroendocrine storage granules, immunohistochemical staining for serotonin, gastrin, insulin, glucagon, and somatostatin was performed using antibodies against these hormones. A monoclonal antibody was used to detect serotonin while polyclonal antisera were used for gastrin, insulin, glucagon, and somatostatin (all from Dako).

For transmission electron microscopy, the specimens were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h and postfixed with 2% osmium tetroxide, dehydrated in alcohol and embedded in Epon. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate. Ultrastructural observation was performed with a JEOL 1200EX electron microscope.

**Establishment of liver metastasis model** Small pieces of tumor tissue, about 5 mm in diameter and weighing  $100 \pm 10$  mg, were resected from subcutaneous EN-1 tumors in the exponential growth phase. These tumor pieces were sutured to the cecal wall of nude mice using 6-0 Dexon (Davis-Geck Inc., Manati, Puerto Rico) after removal of the serosa. To investigate the tumor growth rate of EN-1, tumors were weighed at 3, 4, 5 and 6 weeks after transplantation of small pieces of EN-1 ( $100 \pm 10$  mg,  $n=4$ ; 3, 4, 5 weeks,  $n=8$ ; 6 weeks). The growth rate of EN-1 was expressed as  $Twi/Two$ , where  $Twi$  was the actual tumor weight at a given time and  $Two$  was the initial tumor weight at transplantation. We also investigated whether liver metastasis was macroscopically detectable at a given time.

**Preparation of human VEGF neutralizing monoclonal antibody** Mouse myeloma cells, Sp2/O-Ag14, were fused with spleen cells from BALB/c mice immunized with recombinant human VEGF<sub>121</sub>. BALB/c nude mice were inoculated intraperitoneally with hybridomas, and the monoclonal antibody was purified from the ascitic fluid using a protein-A Sepharose column (Bio-Rad Lab., Richmond, CA).

**Antitumor effect of VEGFAB** To investigate the preventive effect of VEGFAB on liver metastasis of EN-1, 38 mice were divided into two groups: a control group ( $n=19$ ) and a VEGF group ( $n=19$ ). The latter group

received 0.2 ml of VEGFAB at a dose of  $100 \mu\text{g}/\text{mouse}$  intraperitoneally on alternate days from day 10 after tumor transplantation, while the control group received saline. Treatment was continued until the animals were killed at 6 weeks after transplantation. Autopsy was performed immediately, and the cecal tumors were removed and weighed. The metastatic foci were counted by careful macroscopic examination, then the liver of each animal was processed for routine histological examination to confirm the presence of metastases.

**Measurement of tumor tissue VEGF levels** A tumor tissue extract was prepared as described previously.<sup>7)</sup> After samples had been resected from the primary tumors, they were homogenized in phosphate buffer and centrifuged at  $8000g$  for 20 min. VEGF levels were determined by sandwich-type ELISA using anti-human VEGF polyclonal antibody.<sup>8)</sup>

**Statistical analysis** The Student's  $t$  test and the  $\chi^2$  test were used for statistical analysis. A  $P$  value less than 0.05 was considered significant.

## RESULTS

**Morphological and immunohistochemical features of EN-1** Fig. 1a shows an HE-stained section of the original tumor, which is composed of polygonal cells, with occasional ribbon-like structure. There were neither glandular nor acinar structures. The nuclei of the tumor cells showed mild variations in size and mitoses were occasionally present. On the other hand, EN-1 cells also had polygonal cytoplasm and medullary proliferation without specific structures. The degree of hyperchromatism and nuclear variation in size and shape were greater than those of the original tumor cells, and mitotic figures were frequently seen (Fig. 1b). Grimelius' impregnation was strongly positive in the cytoplasm of the original tumor cells (Fig. 2a), and was sparsely positive in the cytoplasm of some EN-1 tumor cells (Fig. 2b).

Ultrastructural examination demonstrated numerous endocrine granules in the cytoplasm of the original tumor cells (Fig. 3a). In EN-1 cells, a few endocrine granules were scattered in the cytoplasm and annulate lamellae were also found in the cytoplasm (Fig. 3b).

These findings demonstrated that EN-1 had lost the typical features of carcinoid and could be classified as endocrine cell carcinoma.

**Growth rate of EN-1** Fig. 4 shows the values of  $Twi/Two$  at 3, 4, 5, and 6 weeks after transplantation.  $Twi/Two$  increased linearly and the weight of the tumor at 6 weeks after transplantation became about 60 times greater than that of the transplanted pieces.

**Liver metastasis of EN-1** Liver metastasis could be identified even at 4 weeks after transplantation in one of 4 mice (Fig. 4). At 6 weeks after transplantation, liver

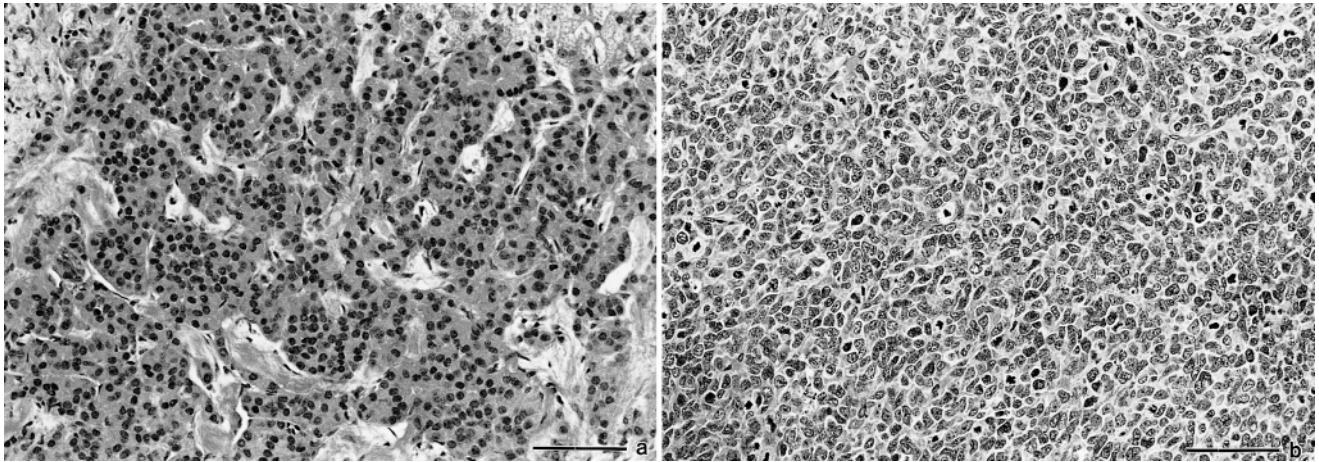


Fig. 1. Histology of the original human carcinoid tumor (a), EN-1 transplanted into the cecal wall of a nude mouse (b). Scale bar=100  $\mu$ m. HE. The original tumor is composed of polygonal cells, occasionally with ribbon-like structure. There is neither glandular nor acinar structure. The nuclei of the tumor cells show mild variations in size and mitoses are occasionally present. On the other hand, EN-1 cells also demonstrate polygonal cytoplasm and medullary proliferation without specific structures. The degree of hyperchromatism and nuclear variation in size and shape is greater than those of the original tumor cells and mitotic figures are frequently seen.

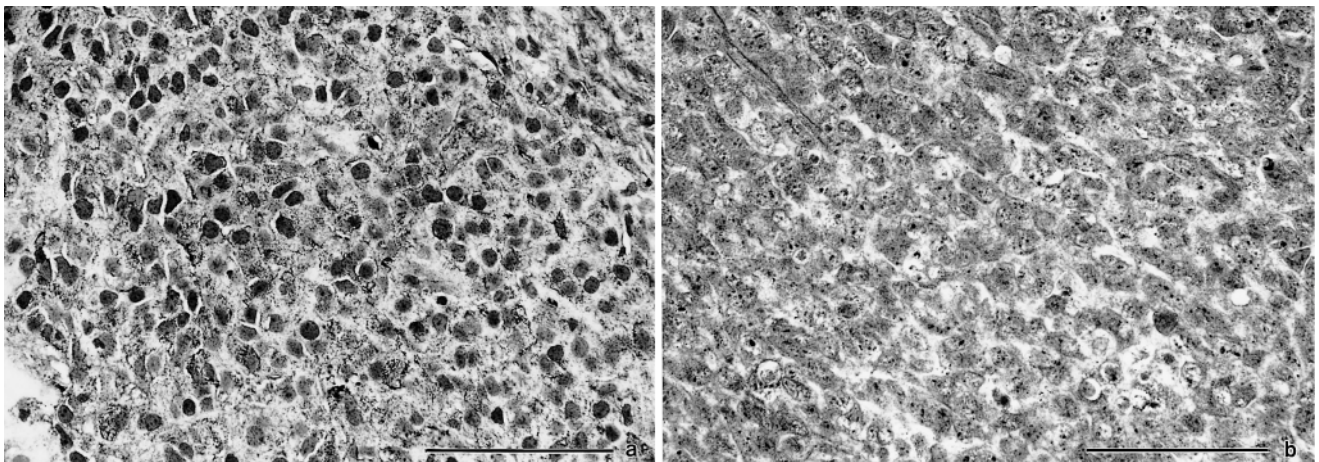


Fig. 2. Grimelius' impregnation of the original tumor (a) and EN-1 transplanted into the cecal wall of a nude mouse (b). Scale bar=100  $\mu$ m. Grimelius' impregnation is strongly positive in the cytoplasm of the original tumor, and is also positive in the cytoplasm in a part of EN-1 tumor cells.

metastasis developed in 87.5% of the mice and metastatic foci could be counted easily. The histological findings of liver metastasis at 6 weeks after transplantation are illustrated in Fig. 5. The metastatic tumor cells were present in the portal area, and occasionally in the sinusoids. The tumor aggregates form medullary masses surrounding the bile ducts. Some mitotic figures were present. These features were identical with those of EN-1.

**Effect of antiVEGFAB on EN-1** Of the 19 control mice, 5 died at 35–40 days after transplantation due to small

bowel obstruction, caused by the huge transplanted cecal tumor. The tumor weight and the number of liver metastatic foci could be evaluated in 3 of these 5 mice, but the other 2 mice were excluded from the experiment because the number of liver metastatic foci could not be counted. All mice of the treated group survived until the end of the experiment.

The cecal tumor weight in the control group at the end of the experiment ranged from 3250 to 7740 mg (mean:  $6316 \pm 2333$  mg). In the treated group, it ranged from 240

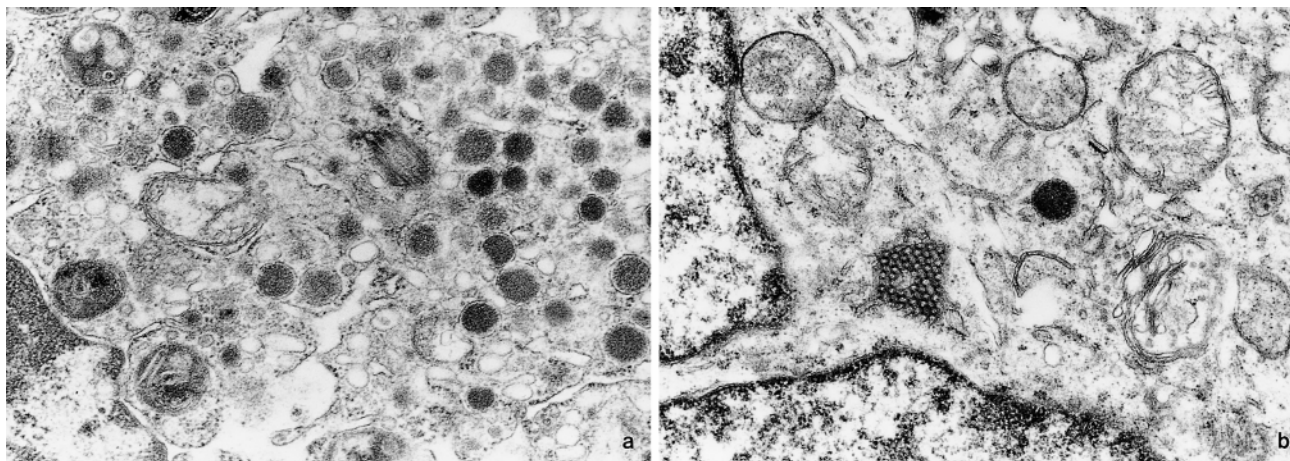


Fig. 3. Ultrastructural examination of the original tumor (a) and EN-1 (b). Numerous endocrine granules were demonstrated in the cytoplasm of the original tumor cell and a few granules were scattered in the cytoplasm of the EN-1 cell.

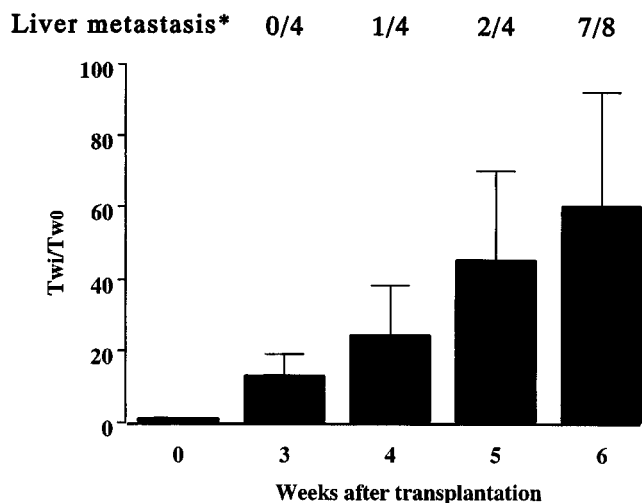


Fig. 4. Growth rate of EN-1 transplanted into the cecal wall. Growth rate is expressed as  $T_{wi}/T_{wo}$ , where  $T_{wi}$  is actual tumor weight at a given time and  $T_{wo}$  is the initial tumor weight at transplantation. The data represent means  $\pm$  SD. The rate of liver metastasis at a given time is expressed as the ratio of the number of mice with liver metastasis to the total number of mice.

to 3480 mg (mean:  $1209 \pm 837$  mg). AntiVEGFAb significantly inhibited the growth of the transplanted tumors ( $P < 0.001$ ) (Table I).

AntiVEGFAb also significantly inhibited the liver metastasis of EN-1 tumors ( $P < 0.01$ ). Liver metastasis was observed in 94.1% of the control mice and in only 10.5% of the treated group (Table I). In addition, massive meta-

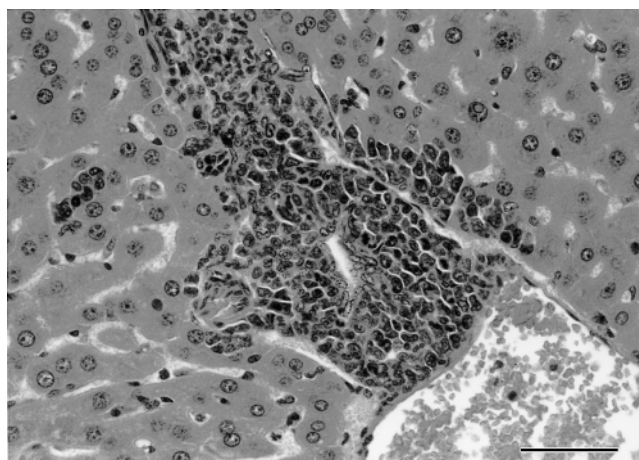


Fig. 5. HE staining of the liver metastasis. Scale bar = 100  $\mu$ m. The metastatic tumor cells are present mainly in the portal area. The tumor aggregates appear as medullary form masses surrounding the bile duct. Some mitotic figures are observed.

static foci (more than 20) were observed in more than 50% of the control mice and in none of the treated mice (Table II).

**VEGF level in the transplanted tumor tissue** The VEGF level in the transplanted tumor was significantly higher in the control group than in the treated group ( $305.1 \pm 174.1$  vs.  $54.7 \pm 41.2$  mg/whole tumor;  $P < 0.0001$ ). The VEGF level in the treated group was 17.9% of that in the control group.

**Body weight** The body weight at the time when the ani-

Table I. Therapeutic Effect of AntiVEGFAB on EN-1

Group	Cecal tumor weight (mg)	No. of mice with liver metastasis
Control (n=17)	6316±2333	16/17
AntiVEGFAB (n=19)	1209±837*	2/19**

\*  $P < 0.001$ , \*\*  $P < 0.01$ , significant difference from the control group.

mals were killed was  $28.52 \pm 1.63$  g in the control group and  $28.44 \pm 1.71$  g in the treated group.

## DISCUSSION

The histological features of EN-1 were more anaplastic than those of the original tumor, which was an atypical carcinoid tumor. The immunohistochemical study also revealed the anaplastic change of EN-1, which showed negative immunohistochemical staining, whereas the original tumor showed positive staining for Chromogranin A, NSE, and somatostatin (data not shown). However, EN-1 was positive for Grimelius' impregnation and ultrastructurally showed evidence of production of endocrine granules. Immunohistochemical examination might have failed to identify the endocrine granules of EN-1 cells because of insufficient antigenicity.

There are two possible scenarios for development of EN-1 cells. Firstly, the original carcinoid tumor cells might have been transformed into EN-1 cells during culture *in vitro*. Secondly, EN-1 cells might have coexisted with the original carcinoid tumor cells and then overcome the resting subpopulations of tumor cells. It was reported that a carcinoid or an adenocarcinoma component was sometimes observed in resected specimens of endocrine neoplasm.<sup>9, 10</sup> The former seems to be applicable to EN-1, because EN-1-like cells were not observed in the original tumor. However, the precise mechanism of the appearance of EN-1 cells is still unknown.

The prognosis of patients with metastatic endocrine neoplasm is poor because no effective therapy has yet been established. For instance, even among gastrointestinal carcinoid tumors, the malignant carcinoid tumors readily metastasize to the liver, and chemotherapy, radiotherapy, and chemoembolization have been employed to treat the liver metastasis of these tumors.<sup>11-14</sup> These modalities were demonstrated to be effective in improving symptoms, but not effective in prolonging the life expectancy.

We investigated the therapeutic effect of an angiogenesis inhibitor, because (i) the solid tumor growth is generally considered to depend on angiogenesis, and the tumor neovascularization is an essential part of the complicated process of metastasis,<sup>15, 16</sup> (ii) EN-1 produced a consider-

Table II. Number of Foci of Liver Metastasis in Control and AntiVEGFAB-treated Mice<sup>a)</sup>

Group	Number of foci			
	0	0 < foci ≤ 10	10 < foci ≤ 20	20 <
Control group	1	3	4	9
AntiVEGFAB group	17	1	1	0

a) Data represents the number of mice.

able amount of VEGF, and (iii) hematogenic metastasis is common with endocrine neoplasms, which may be a suitable target of anti-angiogenic therapy.

To our knowledge, this is the first report of a liver metastatic model of human endocrine neoplasm, although the establishment of carcinoid tumor strains has been reported.<sup>17, 18</sup> Induction of metastasis in nude mice by orthotopic transplantation has already been reported for various neoplasms.<sup>19, 20</sup> The transplantation of "intact tissue," which is considered to maintain better the integrity and characteristics of the original tumor, is more likely to induce distant metastasis. In the present study, EN-1 was transplanted to the cecum, as we considered it technically impossible to perform duodenal transplantation due to lack of space in the mouse peritoneal cavity.

VEGF is a dimeric protein and a mitogen for the endothelial cells of various vessels.<sup>21, 22</sup> VEGF expression has been detected in several human tumors,<sup>23, 24</sup> and VEGF produced by tumor cells binds to specific receptors on endothelial cells and induces endothelial cell proliferation and tube formation.<sup>25, 26</sup> Consequently, it enhances tumor neovascularization. A high level of VEGF mRNA expression was reported to be correlated with that of VEGF protein.<sup>23</sup> Not only VEGF overexpression but also overexpression of VEGF receptors has been detected in colon cancer.<sup>23, 27</sup>

It was also reported that transfection of VEGF into tumor cell lines increased the proliferation rate and metastasis rate.<sup>28</sup> VEGF is a permeability factor which supports tumor growth by improving the supply of nutritional components and oxygen. In addition, hyperpermeability induced by VEGF enhanced angiogenesis.<sup>29</sup> These reports suggest that VEGF may be a good target molecule for anti-angiogenesis therapy.

VEGFAB has been reported to inhibit the proliferation and metastasis of human tumors.<sup>30, 31</sup> In the present study, an antitumor effect of VEGFAB on tumor growth and liver metastases of EN-1 was also clearly demonstrated. Because the ratio (19.1%) of the tumor weight in the treated group to that in the control group was similar to that (17.9%) of the VEGF levels in the two groups, the inhibitory effect of VEGFAB on cecal tumor growth might be mainly mediated by preventing the action of VEGF.

Although the mechanism of inhibition of liver metastasis has not yet been clarified, the therapeutic effect of VEGFAB may be induced by (i) inhibition of angiogenesis in both the primary and metastatic tumors, and (ii) inactivation of some protease(s) induced by VEGF. VEGF was reported to up-regulate plasminogen activator.<sup>32)</sup>

Warren *et al.* reported that in VEGFAB-treated mice neither blood vessels nor expression of VEGF receptor could be demonstrated in liver metastasis of colon cancer induced by splenic-portal injection into nude mice of colon cancer cells.<sup>33)</sup> They suggested that the decrease of VEGF in the circulation might down-regulate the VEGF receptors in the liver. The question then arises, what happens to the tumor when angiogenesis is inhibited? We

observed that experimental administration of an angiogenesis inhibitor, TNP-470, promoted apoptosis of liver metastatic tumor cells.<sup>34)</sup> This suggests that the angiogenesis inhibitor may induce apoptosis, which may render the tumor dormant.

Further studies should be performed to assess the enhancement of the therapeutic effect of VEGFAB by combination with surgery, standard chemotherapeutic agents, or embolization. Issues such as the antigenicity of VEGFAB should also be examined before clinical application.

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