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

Hiroyuki Konno,1 Tomio Arai,2 Tatsuo Tanaka,3 Megumi Baba,1 Keigo Matsumoto,1 Toshikazu Kanai,1 Satoshi Nakamura,1 Shozo Baba,1 Yasuhisa Naito,3 Haruhiko Sugimura,3 Ayako Yukita,4 Makoto Asano4 and Hideo Suzuki4

1Second Department of Surgery, 2First Department of Pathology, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431-3124, 3Department of Pathology, Tokyo Metropolitan Geriatric Hospital, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015 and 4Tsukuba Research Laboratory, Toagousei Co., Ltd., 2 Okubo, Tsukuba 300-2611

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 µg/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±2333 mg (n=17) in the control group and 1209±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±174.1 vs. 54.7±41.2 mg; P<<0.001). VEGFAb did not cause any body weight loss (28.52±1.63 in the control vs. 28.44±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.1) 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.2) 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.3) 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.4–6)

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...
with recombinant human VEGF121. BALB/c nude mice fused with spleen cells from BALB/c mice immunized antibody
Preparation of human VEGF neutralizing monoclonal at a given time.
whether liver metastasis was macroscopically detectable actual tumor weight at transplantation. We also investigated Tw
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 μg/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. After samples had been resected from the primary tumors, they were homogenized in phosphate buffer and centrifuged at 8000 g for 20 min. VEGF levels were determined by sandwich-type ELISA using anti-human VEGF polyclonal antibody.
Statistical analysis The Student’s t test and the χ² test were used for statistical analysis. A P value less than 0.05 was considered significant.
RESULTS
Morphological and immunohistochemical features of EN-1
Preparation of human VEGF neutralizing monoclonal antibody Mouse myeloma cells, Sp2/0-Ag14, were fused with spleen cells from BALB/c mice immunized with recombinant human VEGF121. 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
Effect of VEGFAb on Endocrine Neoplasm

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±2333 mg). In the treated group, it ranged from 240

---

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 µm. 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 µm. 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.
to 3480 mg (mean: 1209±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 metastatic 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±174.1 vs. 54.7±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-
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 neoplasms.9, 10) 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.11–14) 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.15, 16) (ii) EN-1 produced a considerable 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.17, 18) Induction of metastasis in nude mice by orthotopic transplantation has already been reported for various neoplasms.19, 20) 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.21, 22) VEGF expression has been detected in several human tumors,23, 24) and VEGF produced by tumor cells binds to specific receptors on endothelial cells and induces endothelial cell proliferation and tube formation.25, 26) Consequently, it enhances tumor neovascularization. A high level of VEGF mRNA expression was reported to be correlated with that of VEGF protein.27) Not only VEGF overexpression but also overexpression of VEGF receptors has been detected in colon cancer.23, 27)

It was also reported that transfection of VEGF into tumor cell lines increased the proliferation rate and metastasis rate.28) 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.29) These reports suggest that VEGF may be a good target molecule for anti-angiogenesis therapy.

VEGF has been reported to inhibit the proliferation and metastasis of human tumors.30, 31) 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.

### 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±857*               | 2/19**                           |

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

### Table II. Number of Foci of Liver Metastasis in Control and AntiVEGFAb-treated Mice

| Group               | Number of foci |
|---------------------|----------------|
| Control group       | 1 3 4 9        |
| AntiVEGFAb group    | 17 1 1 0       |

a) Data represents the number of mice.
Although the mechanism of inhibition of liver metastasis has not yet been clarified, the therapeutic effect of VEGFαβ 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.23) 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 VEGFαβ by combination with surgery, standard chemotherapeutic agents, or embolization. Issues such as the antigenicity of VEGFαβ should also be examined before clinical application.

(Received May 22, 1998/Revised June 29, 1998/Accepted July 6, 1998)

REFERENCES

1) Creutzfeldt, W. and Stockmann, F. Carcinoids and carcinoma syndrome. Am. J. Med., 82, 4–16 (1987).
2) Bruine, A. P., Diejens, W. N. M., Pijls, M. M. J., Linden, E. P. M. v.d., Rouusch, M. J. M., Moerkert, P. T., de Goei, A. F. P. M. and Bosman, F. T. NCI-H716 cells as a model for endocrine differentiation in colorectal cancer. Virchows Arch. B Cell Pathol., 62, 311–320 (1992).
3) Oberg, K. Endocrine tumors of the gastrointestinal tract: systemic treatment. Anticancer Drugs, 5, 503–519 (1994).
4) Tanaka, T., Konno, H., Matsuda, I., Nakamura, S. and Baba, S. Prevention of hepatic metastasis of human colon cancer by angiogenesis inhibitor TNP-470. Cancer Res., 55, 836–839 (1995).
5) Konno, H., Tanaka, T., Kanai, T., Maruyama, K., Nakamura, S. and Baba, S. Efficacy of an angiogenesis inhibitor, TNP-470, in xenotransplanted human colorectal cancer with high metastatic potential. Cancer, 77, 1736–1740 (1996).
6) Kanai, T., Konno, H., Tanaka, T., Matsumoto, K., Baba, M., Nakamura, S. and Baba, S. Effect of angiogenesis inhibitor TNP-470 on the progression of human gastric cancer xenotransplanted into nude mice. Int. J. Cancer, 71, 838–841 (1997).
7) Takano, S., Yoshii, Y., Kondo, S., Suzuki, H., Maruno, T., Shirai, S. and Nose, T. Concentration of vascular endothelial growth factor in the serum and tumor tissue of brain tumor patient. Cancer Res., 56, 2185–2190 (1996).
8) Kondo, S., Asano, M., Matsuo, K., Ohmori, I. and Suzuki, H. Vascular endothelial growth factor/vascular permeability factor is detectable in the sera of tumor-bearing mice and cancer patients. Biochim. Biophys. Acta, 1221, 211–214 (1994).
9) Arai, T. and Kino, J. Histochemical and ultrastructural analysis of glandular differentiation in typical carcinoid tumor of the hindgut. Pathol. Int., 44, 49–56 (1994).
10) Matui, K., Kitagawa, M., Miwa, A., Kuroda, Y. and Tsuji, M. Small cell carcinoma of the stomach: a clinicopathologic study of 17 cases. Am. J. Gastroenterol., 86, 1167–1175 (1991).
11) Kvoles, L. K. and Buck, M. Chemotherapy of metastatic carcinoid and islet cell tumors. A review. Am. J. Med., 82, 77–83 (1987).
12) Ruszniewski, P., Rougier, P., Roche, A., Legmann, P., Sibert, A., Hochaf, S., Ychou, M. and Mignon, M. Hepatic arterial chemoembolization in patients with liver metastases of endocrine tumors. A prospective phase II study in 24 patients. Cancer, 71, 2624–2630 (1993).
13) Therasse, E., Breittmayer, F., Roche, A., De Baere, T., Indushekar, S., Deurex, M., Lasser, P., Elias, D. and Rougier, P. Transcatheter chemoembolization of progressive carcinoid liver metastasis. Radiology, 189, 541–547 (1993).
14) Chakravarthy, A. and Abrams, R. A. Radiation therapy in the management of patients with malignant carcinoid tumors. Cancer, 75, 1386–1390 (1995).
15) Folkman, J. Anti-angiogenesis: new concept for therapy of solid tumor. Ann. Surg., 175, 409–416 (1972).
16) Folkman, J. What is the evidence that tumours are angiogenesis dependent? J. Natl. Cancer Inst., 82, 4–6 (1990).
17) Ganen, Y. J., Launay, J. M., Debons-Guillemin, M. C., Lasneret, J., Roucayrol, A. M., Lesser, J., Peries, G. and Dreux, C. First heterotransplantation of a human carcinoid tumor into nude mice. Cancer, 68, 893–902 (1991).
18) Evers, B. M., Townsend, C. M., Jr., Upp, J. R., Allen, E., Hurlibut, C., Kim, S. W., Rajaraman, S., Singh, P., Reubi, J. C. and Thompson, J. C. Establishment and characterization of a human carcinoid in nude mice and effect of various agents on tumor growth. Gastroenterology, 101, 303–311 (1991).
19) Morikawa, K., Walker, S. M., Jessup, J. M. and Fidler, I. J. In vivo selection of highly metastatic cells from surgical specimens of different primary human colon carcinomas implanted into nude mice. Cancer Res., 48, 1943–1948 (1988).
20) Fu, X., Besterman, J. M., Monosov, A. and Hoffman, R. M. Models of human metastatic colon cancer in nude mice.
orthotopically constructed by using histologically intact patient specimens. Proc. Natl. Acad. Sci. USA, 88, 9345–9349 (1991).

21) Leung, D.W., Cachianes, G., Kuang, W. J., Goeddel, D.V. and Ferrara, N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science, 246, 1306–1309 (1989).

22) Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J. and Connolly, D. T. Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science, 246, 1309–1312 (1989).

23) Brown, L. F., Berse, B., Jackman, R. W., Tognazzi, K., Manseau, E. J., Senger, D. R. and Dvorak, H. F. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. Cancer Res., 53, 4727–4735 (1993).

24) Plate, K. H., Breier, G., Weich, H. A. and Risau, W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature, 359, 845–848 (1992).

25) De Vries, C., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N. and Williams, L. T. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science, 255, 989–991 (1992).

26) Terman, B. I., Dougher-Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, D. C., Gospodarowicz, D. and Bohlen, P. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial growth factor. Biochem. Biophys. Res. Commun., 187, 1579–1586 (1992).

27) Takashashi, Y., Kitadai, Y., Bucana, C. D., Cleary, K. R. and Ellis, L. M. Expression of vascular endothelial growth factor and its receptor (KDR) correlates with vascularity, metastasis, and proliferation of colon cancer. Cancer Res., 55, 3964–3968 (1995).

28) Claffey, K. P., Brown, L. F., del Aguila, L. F., Tognazzi, K., Yeo, K. T., Manseau, E. J. and Dvorak, H. F. Expression of vascular permeability factor/vascular endothelial growth factor by melanoma cells increases tumor growth, angiogenesis, and experimental metastasis. Cancer Res., 56, 172–181 (1996).

29) Dvorak, H. F., Brown, L. F., Detmar, M. and Dvorak, A. M. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am. J. Pathol., 146, 1029–1039 (1995).

30) Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S. and Ferrara, N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature, 362, 841–844 (1993).

31) Asano, M., Yukita, A., Matsumoto, T., Kondo, S. and Suzuki, H. Inhibition of tumor growth and metastasis by an immunoneutralizing monoclonal antibody to human vascular endothelial growth factor/vascular permeability factor 121. Cancer Res., 55, 5296–5301 (1995).

32) Mandriota, S. J., Seghezzi, G., Vasalli, J. D., Ferrara, N., Wasi, S., Mazzieri, R., Mignatti, P. and Pepper, M. S. Vascular endothelial growth factor increases urokinase receptor expression in vascular endothelial cells. J. Biol. Chem., 270, 9709–9716 (1995).

33) Warren, R. S., Yuan, H., Matli, M. R., Gillett, N. A. and Ferrara, N. Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. J. Clin. Invest., 95, 1789–1797 (1995).

34) Ahmed, M. H., Araii, T., Konno, H., Nahar, L., Tanaka, T., Izumiya, N., Takubo, K., Nakamura, S. and Baba, S. Regression of metastatic liver tumors in rats treated with angiogenesis inhibitor TNP-470: occurrence of apoptosis and necrosis. Jpn. J. Cancer Res., 88, 977–981 (1997).